UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

UNAM POSGRADO

FACULTAD DE QUÍMICA

PROGRAMA DE MAESTRÍA Y DOCTORADO EN CIENCIAS BIOQUÍMICAS

MECANISMO DE RESISTENCIA A CADMIO MEDIADO POR TIOLES EN *Euglena gracilis*

T E S I S

QUE PARA OBTENER EL GRADO DE :

DOCTOR EN CIENCIAS (BIOQUÍMICAS)

P R E S E N T A :

BIOL. DAVID GUILLERMO MENDOZA COZATL

Tutor: DR. RAFAEL MORENO SÁNCHEZ

MÉXICO, D. F. Agosto 2005

MECANISMO DE RESISTENCIA A CADMIO MEDIADO POR TIOLES EN *Euglena gracilis*

RECONOCIMIENTOS

Esta tesis doctoral se realizó bajo la dirección del Dr. Rafael Moreno Sánchez en el Departamento de Bioquímica del Instituto Nacional de Cardiología "Ignacio Chávez"

El Comité Tutoral que asesoró el desarrollo de esta tesis estuvo formado por:

Dr. Rafael Moreno Sánchez	Instituto Nacional de Cardiología
Dr. Juan Pablo Pardo Vázquez	Facultad de Medicina, UNAM
Dr. Rogelio Rodríguez Sotres	Facultad de Química, UNAM

Se reconoce la colaboración del Dr. Guillermo Mendoza Hernández, del Departamento de Bioquímica de la Facultad de Medicina, UNAM, en cuyo laboratorio se llevó a cabo la determinación de aminoácidos en muestras de *Euglena gracilis*.

El proyecto fue apoyado parcialmente por CONACYT (43811-Q), DGAPA-UNAM (PAPIIT-IN215705) y PAEP-UNAM (203319). Durante los estudios de doctorado, se le otorgó a David G. Mendoza Cózatl una beca de CONACYT (no. 144638) y una complementaria de DGEP-UNAM.

El Jurado de Examen Doctoral estuvo constituido por:

Presidente	Dr. Francisco Xavier Soberón Mainero	Instituto de Biotecnología, UNAM
Vocal	Dr. Juan Pablo Pardo Vázquez	Facultad de Medicina, UNAM
Vocal	Dr. Mario Luis Calcagno Montans	Facultad de Medicina, UNAM
Vocal	Dr. Guillermo Mendoza Hernández	Facultad de Medicina, UNAM
Secretario	Dr. Edmundo Chávez Cossío	Instituto Nacional de Cardiología
Suplente	Dr. Juan Luis Rendón Gómez	Facultad de Medicina, UNAM
Suplente	Dr. Arturo Navarro Ocaña	Facultad de Química, UNAM

.....para Norma y Mariana

Alguna vez creí saber de que se trataba esto de la vida, pero llegaron ustedes y me hicieron ver que había mucho más de lo que yo pude haber imaginado.....

Sebastián y los que vienen están incluidos en esta dedicatoria, por que ustedes están en nuestras mentes.

Dedicatorias

A mi madre, esa mujer incansable que aún después de tantos años me sigue sorprendiendo. Sergio, Mini y Nizy. Papá, hermanito y hermanita, mi pequeña gran familia...

A Giorgio y Ofe, esos dos abuelos que solo supieron apoyar y consentir a este nieto ...

Carlos (pirinolo !), Carla, Abril, Martha (la bruja !) y en realidad a todos los Cózatl, por ser una gran familia...

Para el tío Bibiano (q.e.p.d.) y la tía Lupita, por el apoyo que siempre nos dieron, a mí, a mi mamá y a mi familia...

A la familia Castro-Guerrero, mi nueva familia, Lulú (mamá), Lulú (hija), Andrés (senior y junior), Luisillo !

A la familia Mendoza, la otra gran parte de la historia, y cuyo apoyo también estuvo presente...

A Paco y Moni, gracias por aceptar junto con nosotros ese gran reto de sacar adelante a nuestros hijos (y a Sophi también por supuesto).

Agradecimientos

Mi más sincero y profundo agradecimiento al Dr. Rafael Moreno Sánchez, por haberme dado la oportunidad de desarrollarme como Investigador del Departamento de Bioquímica del Instituto Nacional de Cardiología. Además de apoyarme tanto académica como personalmente.

Al Dr. Edmundo Chávez Cossío por el apoyo brindado, personal y académico, durante toda mi estancia en el Instituto Nacional de Cardiología.

Y gracias a todos los integrantes del Departamento de Bioquímica, Jassito, Jimena, José, Israel (chiquitín !), Fernando, Fernanda, J.J., Alvarito, Cesar (q.e.p.d.), Rus, Luz, Erika, Emma, Sara, Fernanda, Lalo, Mohammed, Natalia, Silvia, Ceci, Noemí, Gerardo, de verdad gracias a todos ustedes!!!

Y a los buenos amigos de toda la vida, Jesús, Paco, Viorel, Fernando (Chino)....

.. y a Dios, por dejarnos entrever sus ideas y sus inventos.

"Mecanismo de resistencia a cadmio mediado por tioles en *Euglena gracilis*"

Resumen

Euglena gracilis es un protista fotosintético con una elevada capacidad de resistencia y acumulación de cadmio (Cd^{2+}). Como parte del mecanismo de resistencia, *Euglena* sintetiza polímeros de glutatión (GSH), denominados fitoquelatinas (FQs), los cuales son capaces de secuestrar e inactivar al Cd^{2+}. En plantas y algunas levaduras, el mecanismo de resistencia y acumulación de Cd^{2+} mediado por GSH y FQs consiste en la compartamentalización de ambos compuestos dentro de la vacuola. El Cd^{2+} puede ser transportado a la vacuola como ion libre o en forma de complejo FQ-Cd ó GS_2-Cd, ambos procesos son dependientes de energía y catalizados por transportadores independientes. Dentro de la vacuola, los complejos FQ-Cd, el Cd^{2+} libre y el sulfuro forman complejos de alto peso molecular (HMWC; ≈ 30 kDa) alrededor de un núcleo de CdS, los cuales inactivan y mantienen fuertemente unido al Cd^{2+}.

Euglena gracilis carece de una vacuola tipo planta y mas del 60% de las FQs y el Cd^{2+} se localizan en el cloroplasto (Mendoza-Cózatl et al., 2002), lo que nos llevó a proponer un modelo de compartamentalización en el cual el Cd^{2+} puede entrar al cloroplasto como ion libre y unido a GSH, FQs o ambos. Además, las FQs también podrían sintetizarse en el interior de este organelo, a partir de GSH endógeno. Puesto que la inactivación completa del Cd^{2+} requiere de la formación de HMWC, pensamos que estos complejos pueden estar presentes en el cloroplasto de *Euglena*.

La presente tesis se centró en los siguientes objetivos:

1. Caracterizar algunos de los eventos involucrados en el mecanismo de resistencia propuesto: (a) entrada de Cd^{2+} al cloroplasto como ion libre, (b) localización intracelular de la fitoquelatina sintetasa y (c) caracterización de los HMWC de *Euglena*.

2. Analizar la capacidad de *Euglena gracilis* de acumulación y remoción de Cd^{2+} del medio de cultivo en presencia de diferentes metales pesados.

Los resultados obtenidos para el primer objetivo muestran que el Cd^{2+} puede entrar al cloroplasto de *Euglena* en forma de ion libre mediante dos procesos: difusión facilitada (cinética saturable) y difusión simple (cinética lineal). Los parámetros cinéticos que se obtuvieron para el transporte fueron una *Vm* de 11 nmol Cd^{2+} min^{-1} (mg proteína)$^{-1}$ y una Km_{Cd} de 13 µM para el componente saturable, mientras que el componente lineal presentó una pendiente de 0.13 nmol Cd^{2+} min^{-1} (mg proteína)$^{-1}$ μM^{-1}, que representa el incremento en la velocidad de captación de Cd^{2+} por cada µmol Cd^{2+}/L presente en el ensayo. El transporte de Cd^{2+} no se afectó por la adición de desacoplantes, inhibidores de la fotosíntesis, exposición a la luz u oscuridad. Sin embargo, el transporte se inhibió por K^+ o por baja temperatura (4°C). Los cloroplastos de las células cultivadas con 50 µM $CdCl_2$ (cloroplastos ZCd_{50}), mostraron un incremento del 60% en la *Vm* del transportador [11.2 vs 18.1, nmol Cd^{2+} min^{-1} (mg proteína)$^{-1}$], mientras que la *Km* y la velocidad del componente lineal permanecieron constantes. Las propiedades cinéticas del transporte de Cd^{2+} sugieren que el transportador podría pertenecer a las familias de transportadores CDF (Cation Difussion Facilitator) ó ZIP (Zinc-Iron protein) (Mendoza-Cózatl y Moreno-Sánchez, 2005).

Por otro lado, la retención del Cd^{2+} en los cloroplastos correlacionó con la cantidad de tioles y sulfuro. Los cloroplastos ZCd_{50} contenían 4 veces más tioles y sulfuro que los cloroplastos control y retuvieron 6 veces más Cd^{2+} que estos últimos. Además, determinamos que las FQs pueden sintetizarse en el interior del cloroplasto y que aproximadamente el 39% de la fitoquelatina sintetasa de *Euglena gracilis* se localiza dentro de este organelo. Las fitoquelatinas del cloroplasto se localizaron en su mayoría en

la fracción soluble de alto peso molecular (> 50 kDa), lo cual sugiere que la formación de HMWC está relacionada con la resistencia e inactivación del Cd^{2+} en *Euglena gracilis* (Mendoza-Cózatl et al., 2005). Estos complejos se aislaron a partir de células enteras, y se obtuvo un compuesto homogéneo de 148 kDa. Su caracterización fisicoquímica reveló que difieren significativamente de los HMWC encontrados en plantas y levaduras respecto a su peso molecular y su composición, tanto de péptidos como de aminoácidos. Sin embargo, la estequiometría tiol+sulfuro/Cd^{2+} de 3.4 coincide con la encontrada en plantas y levaduras, lo cual también sugiere que estos complejos están involucrados en la inactivación y acumulación de Cd^{2+} en *Euglena gracilis* (Mendoza-Cózatl et al., 2005).

Finalmente, se evaluó la capacidad de *Euglena* para remover Cd^{2+} del medio de cultivo variando la concentración de Zn^{2+} y Pb^{2+}. *Euglena* fue capaz de remover hasta el 80% del Cd^{2+} cuando se cultivó en presencia de 20 y 50 µM $CdCl_2$. Concentraciones mayores de Cd^{2+} disminuyen drásticamente el crecimiento por lo que, a pesar de incrementarse la captación de Cd^{2+} por célula, disminuye la capacidad de remoción total del cultivo. El (5-300 µM) no tuvo efecto sobre la capacidad de remoción del Cd^{2+} ni afectó el contenido de tioles no proteicos (Cys, GSH, FQs). Por otro lado, el Pb^{2+} (100 y 200 µM) disminuyó la capacidad de remoción de Cd^{2+}, a pesar de tener un efecto aditivo en el incremento en la concentración de tioles inducido por Cd^{2+}, sugiriendo que ambos metales comparten el mecanismo de resistencia y acumulación mediado por tioles.

Abstract

Euglena gracilis is a photosynthetic protist with a high cadmium (Cd^{2+}) resistance and accumulation capacity. As part of the Cd^{2+} resistance mechanism, *Euglena* synthesizes phytochelatins (FQs), glutathione (GSH) derived peptides, which chelate and inactivate Cd^{2+}. In plants and some yeast, the phytochelatin-GSH mediated Cd^{2+} resistance mechanism involves the compartmentation of Cd^{2+} and the FQ-Cd complexes (or GS_2-Cd complexes) into the vacuole. Both processes are energy-dependent and catalyzed by independent transporters. Once inside the vacuole, FQ-Cd or GS_2-Cd complexes, free Cd^{2+} and sulfide form high molecular weight complexes (HMWC; 30 kDa) around a CdS crystallite core which maintain Cd^{2+} tightly bound.

Euglena gracilis lacks a plant-like vacuole and more than 60% of the Cd^{2+} and FQs are located inside the chloroplast; these facts led us to propose a compartmentation model in which Cd^{2+} may enter into the chloroplast as a free ion and bound to FQs or GSH. In addition, and because GSH is present inside the chloroplast, FQs could be also synthesized inside the organelle. Because full Cd^{2+} inactivation requires HMWC formation, we proposed that these complexes may also be formed inside the *Euglena* chloroplast.

This thesis was focused on the following objectives:

1.- From the model described above, (a) characterization of Cd^{2+} transport as a free ion into the chloroplast, (b) intracellular location of phytochelatin synthase and (c) characterization of *Euglena* HMWC.

2.- Determine the ability of *Euglena* to remove Cd^{2+} form the culture media in the presence of different heavy metals.

The results obtained demonstrated that Cd^{2+} can enter into the chloroplast as a free ion through two processes: facilitated diffusion (saturable kinetics) and simple diffusion (linear kinetics). Kinetic parameters obtained for the transporter were *Vm* 11 nmol Cd^{2+} min^{-1} (mg protein)$^{-1}$ and Km_{Cd} of 13 μM, whereas the linear component showed a slope of 0.13 nmol Cd^{2+} min^{-1} (mg protein)$^{-1}$ μM^{-1} which represents the increase in Cd^{2+} uptake for each μmol per liter in the assay. Cd^{2+} transport was not affected by uncouplers, photosynthesis inhibitors, light or darkness. However, Cd^{2+} transport was inhibited by K^+ and low temperature (4ºC). Chloroplasts isolated from cells grown in the presence of 50 μM $CdCl_2$ showed a 60% increase in the *Vm* of the transporter without affecting the *Km* or the uptake rate through the linear component. The kinetic properties of the Cd^{2+} uptake into the chloroplast suggested that the transporter involved may belong to the CDF (Cation Diffusion Facilitator) or ZIP (Zinc-Iron Proteins) transporter families (Mendoza-Cózatl and Moreno-Sánchez, 2005).

On the other hand, Cd^{2+} retention in chloroplasts was related with the thiols and sulfide content. Chloroplasts ZCd_{50} contained 4-fold more thiols and sulfide than control chloroplasts and retained 6-fold more Cd^{2+} than the later ones. In addition, FQs may be synthesized in the chloroplasts and approximately 39% of the phytochelatin synthase was located inside this organelle. Along this line, the FQs found in the chloroplast were located mostly in the high molecular weight fraction (>50 kDa), suggesting that HMWC may be part of the Cd^{2+} resistance-inactivation process in *Euglena gracilis* (Mendoza-Cózatl et al., 2005). These complexes were purified to homogeneity as a 148 kDa compound. Their physicochemical characterization revealed significant differences from the HMWC

isolated from plants and some yeast, regarding molecular weight, amino acid and peptide composition. However, the thiol+sulfide/Cd^{2+} ratio of 3.4 agreed with that reported for plants and yeast, suggesting that these complexes are involved in the Cd^{2+} accumulation-inactivation process in *Euglena gracilis*.

Finally, the ability of *Euglena* to remove Cd^{2+} from the culture medium in the presence Zn^{2+} and Pb^{2+} was evaluated. *Euglena* was able to remove up to 80 % of the Cd^{2+} present in the medium when cultured in the presence of 20 and 50 μM $CdCl_2$. Higher concentrations, although increased the Cd^{2+} uptake *per* cell, severely decreased the cell density of the culture, thus diminishing the overall removal capacity of the culture. Zn^{2+} (5-300 μM) showed no effect on the Cd^{2+} accumulation or content of non-proteic thiols (Cys, GSH, FQs). On the other hand, Pb^{2+} (100 and 200 μM) decreased the Cd^{2+} removal ability of *Euglena*. This was in contrast to the increase in the thiol content promoted by Pb^{2+}, suggesting that both heavy metals share the thiol-mediated accumulation mechanism.

INDICE **página**

Capitulo 1. Los metales y su clasificación.

1.1 *Los metales en la evolución*

Hace aproximadamente 4,500 millones de años que este planeta se formó y junto con él, se formaron la mayoría de los elementos que conocemos actualmente. De los más de 100 elementos que conforman la Tabla Periódica, sólo 30 fueron seleccionados para formar parte de las células (Lehninger et al., 2000). Durante el transcurso de la evolución, las condiciones de la Tierra se han modificado drásticamente, de reductoras a oxidantes, convirtiendo elementos que antes eran inaccesibles para los organismos (metales en forma de sulfuros insolubles) en compuestos solubles (sulfatos) y, por lo tanto, accesibles para las células (Clarkson, 1995). Se piensa que esta fue la principal razón por la cual los organismos no sólo tuvieron que contender con una "tormenta" de nuevos elementos, principalmente metales y oxígeno, sino que además los organismos evolucionaron de tal manera que han aprovechado sus propiedades para incluirlos como parte esencial del metabolismo celular (Clarkson, 1995).

Los elementos más importantes (99%) que constituyen a los seres vivos son C, H, O, N, S, P. El 1% restante comprende al menos 20 elementos, algunos de ellos denominados "traza", entre los cuales se encuentran el sodio, potasio, magnesio, calcio, manganeso, hierro, cobalto, cobre y zinc (Lehninger et al., 2000). Es interesante notar que, hablando particularmente de metales, sólo aquellos de mayor abundancia en los océanos fueron los que se incorporaron al metabolismo celular y a los cuales se les ha denominado metales *esenciales* (Tabla 1.1).

Existe otra variedad de metales, relativamente poco abundantes en la naturaleza como el oro, la plata, el cadmio, el mercurio y el plomo, que carecen de función biológica y por lo tanto se han denominado *no esenciales* (Tabla 1.1). Sin embargo, los metales *no*

esenciales pueden compartir características fisicoquímicas con los metales *esenciales*, de tal manera que los sistemas de transporte de un organismo pueden ser incapaces de distinguirlos y, por lo tanto, incorporar ambos tipos de metales a la célula (Guerinot, 2000; Williams et al., 2000). Una vez dentro, los metales *no esenciales* usurparan las funciones de los metales *esenciales*, teniendo como resultado un efecto tóxico para la célula. Este fenómeno de toxicidad no es único de los iones metálicos.

Tabla 1.1 Concentración de algunos metales (µg / ml) en cuerpos de agua no contaminados

Metal	**Aguas Intracontinentales**	**Aguas Oceánicas**
Esenciales		
Cobalto[a]	< 0.1	< 0.007
Cobre	0.2-2	0.32-0.57
Hierro	----	0.014-0.028
Zinc	0.5-5	0.6-1
No esenciales		
Plata	----	0.00004-0.0025
Cadmio	0.005-0.05	0.00016-0.124
Cromo	0.1-0.5	0.00016-0.05
Mercurio	< 0.0012	0.001-0.005
Níquel	0.01-1	0.05-0.65
Plomo	0.05-0.5	0.001-0.015
Vanadio	< 0.5	0.0046-0.55

[a] La valencia de cada elemento no fué especificada. Tabla modificada de Moreno-Sánchez y Devars, 1999.

Oxianiones tales como el arsenato (AsO_4^{3-}) o el cromato (CrO_4^{2-}) pueden interferir en el metabolismo relacionado con el fosfato (PO_4^{3-}) y el sulfato (SO_4^{2-}), respectivamente

(Ohtake et al., 1987; Gorby, 1988; Cervantes et al., 2001). Por otra parte, resulta también interesante que los elementos menos abundantes y, por lo tanto, los menos accesibles a los organismos, son los de mayor toxicidad para la mayoría de los sistemas biológicos (Tabla 1.1; ver más adelante). Independientemente de si un metal es *esencial* o no, y debido a la reactividad de los metales con las biomoléculas, un incremento en la concentración intracelular de ambos tipos de metales resulta tóxica para las células, por lo que estas deben tener mecanismos que regulen finamente la concentración intracelular de los metales *esenciales* y otros que discriminen e inactiven a los metales *no esenciales*.

1.2 *Nomenclatura de metales*

La mayor parte de la literatura científica actual acepta, sin mayor argumentación, la distinción entre metales *esenciales* y *no esenciales*. Los primeros son indispensables para alguna función biológica, los segundos no. Adicionalmente, la literatura científica maneja muy frecuentemente el término "metal pesado". Existen al menos 10 diferentes definiciones de "metal pesado" y la mayoría están relacionadas con la densidad de los elementos (revisado por Duffus, 2002). Hay autores que se refieren a los "metales pesados" como aquellos elementos con una elevada densidad (> 3.5 g/ml), siendo 5 g/ml la densidad usada con mayor frecuencia (Parker, 1989; Lozet y Mathieu, 1991; Morris, 1992). Sin embargo, diversos autores y agrupaciones con autoridad científica (la Unión Internacional para la Química Pura y Aplicada, IUPAC, por ejemplo) han externado su preocupación acerca de la ambigüedad del termino "metal pesado" y de la falta de seriedad con la que se ha evaluado la "pesadez" de un elemento (Nieboer and Richardson, 1980; Duffus, 2002). Históricamente, también se ha relacionado el término "metal pesado" con la toxicidad biológica y el daño ambiental. Sin embargo, el grado de

toxicidad de un elemento o el riesgo para un ecosistema no está relacionada de ninguna manera con la densidad o "pesadez" de un elemento (Duffus, 2002).

Una clasificación alternativa para metales, basada en la reactividad de cada metal con los principales grupos funcionales de las biomoléculas, consiste en agruparlos de acuerdo con sus características fisicoquímicas. Esta clasificación se basa en el comportamiento de los metales como ácidos de Lewis (aceptores de electrones) y estaría conformada por 3 grupos principales: clase A o ácidos duros, clase B ó ácidos suaves y clase intermedia (Tabla 1.2.1; Duffus, 2002). De acuerdo con esta clasificación, los metales de la clase A tienen preferencia por moléculas con oxígeno, los metales de la clase B muestran afinidad por moléculas con nitrógeno y/o azufre y los de la clase intermedia no muestran una afinidad distintiva por algún grupo en particular (Duffus, 2002). Sin embargo, hay que notar que los metales de la clase A pueden diferenciarse fácilmente por sus propiedades respecto a los de las clases B e intermedia, mientras que en estas dos últimas las diferencias no son tan obvias. De hecho diversos autores, usando esta misma clasificación, han colocado al Cd^{2+} en la clase intermedia, mientras que la IUPAC lo ha colocado en la clase B (Nieboer y Richardson, 1980, Duffus, 2002).

Por otro lado, la clasificación de metales de acuerdo a su reactividad, aunque sistemática y basada en propiedades fisicoquímicas, puede no reflejar o anticipar el grado de toxicidad de un metal hacia un organismo o ecosistema. Por ejemplo, para la mayoría de los organismos, el orden que siguen los iones metálicos en cuanto a su toxicidad está bastante bien conservado: $Hg^{2+} > Ag^{1+} > Cu^{2+} > Cd^{2+} > Pb^{2+} > Zn^{2+}$ (compilado por Nieboer and Richardson, 1980; Devars et al., 1999). De acuerdo con este orden, parecería claro que los metales pertenecientes a la clase B (metales suaves) son los más tóxicos, y que esta toxicidad estaría relacionada con su interacción con los grupos tioles (-

SH) o amino (-NH_3) de las biomoléculas. Sin embargo, tomando como ejemplo al Cd^{2+}, dos de sus efectos más estudiados son el desplazamiento del Ca^{2+} del complejo Ca^{2+}-calmodulina y el desplazamiento del Na^+ y del Ca^{2+} de monómeros de fosfatidilserina y fosfatidiletanolamina, siendo esta última una de las bases para explicar el daño a las membranas biológicas (Vallee y Ulmer, 1972; Díaz-Barriga, 1991). Como se muestra en la tabla 1.2.1, tanto el Ca^{2+} como el Na^{2+} pertenecen a la clase A de metales, por lo que no habría una relación estricta entre la clase del metal y el mecanismo de toxicidad del metal.

Tabla 1.2.1 Clasificación de metales de importancia biológica (esenciales y no esenciales) según la IUPAC.

	Aceptores de electrones de tamaño pequeño y poco polarizables[a]
Clase A Metales duros	Li^+, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, Fe^{3+}, Sr^{2+}, Cs^+, Ba^{2+}, La^{2+},Tl^+.
	Aceptores de electrones de mayor tamaño y muy polarizables[a]
Clase B Metales suaves	Cu^+, Ag^+, Cd^{2+}, Au^+, Hg^{2+}, Pb^{2+}.
Clase intermedia	Cr^{6+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{4+}.

[a] Respecto a la capacidad de deformación de su nube electrónica. Modificada de Duffus, 2002.

Otro punto importante a considerar es la valencia del elemento. Dependiendo de su estado de oxidación, el plomo, el hierro y el cobre pueden ser colocados en diferentes clases: Fe^{3+} (clase A), Cu^{1+} y Pb^{2+} (clase B) y Fe^{2+}, Cu^{2+}, y Pb^{4+} (clase intermedia). La especie química del ligando también juega un papel importante en la estabilidad de un complejo ligando-metal, así como el número de grupos funcionales que interactúan con el metal. La tabla 1.2.2 muestra diferentes constantes de estabilidad (K_{est}, el inverso de la constante de disociación) para el Cd^{2+} y algunos compuestos. Hay que notar que, incluso

si se trata de ligandos con el mismo grupo funcional, por ejemplo cisteína y glutatión, las K_{est} varían significativamente (4 veces), mientras que la diferencia en afinidad por el sulfuro es aún mayor (Tabla 1.2.2). Esto se debe a que en el caso del glutatión y compuestos

Tabla 1.2.2 Constantes de estabilidad del Cd^{2+} por diferentes ligandos.

Ligando	Log K_{est}
Cys	9.89
GSH	10.5
Sulfuro	6.1
Citrato	9.44, 5.56[a]
Malato	2.36, 1.34
Glutamato	4.78, 2.78
Lactato	1.54, 0.87
Pirofosfato	5.6
EGTA	17.53, 11.30
EDTA	17.39, 9.88

[a] En los casos donde se muestran dos valores de K_{est}, estos se refieren a diferentes estados de protonación del ligando, el primero corresponde al ligando totalmete desprotonado y el segundo con un protón asociado. Valores compilados por Sillén y Martell, 1971.

homólogos como las FQs (ver mas adelante), el Cd^{2+} interactúa tanto con el grupo α-carboxilato del glutámico como con el tiol, mientras que con la cisteína el Cd^{2+} solo interactúa con el tiol (Satofuka et al., 2001). Este fenómeno ocurre también en compuestos con oxígeno. Por ser de clase B, se esperaría que el Cd^{2+} tuviera más afinidad por compuestos con azufre o nitrógeno, pero como se muestra en la tabla 1.2.2

las moléculas con mayor afinidad por Cd^{2+} son el EDTA y el EGTA; la razón es que más de un carboxilato está involucrado en la unión del metal.

Los radios iónicos es otra propiedad de los elementos que podría usarse como herramienta para entender, al menos en parte, la competencia y la toxicidad de metales hacia una función biológica. A pesar de que el radio iónico de un elemento varía de acuerdo con su valencia, se puede tener una idea de cómo varía el radio iónico de un elemento de acuerdo a su posición en la tabla periódica (Casabó, 1999). Por ejemplo:

1) Los radios iónicos aumentan gradualmente hacia abajo en un grupo de la tabla periódica: $Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$.
2) Los radios de los iones con la misma carga disminuyen a lo largo de un periodo de la tabla periódica: $Ca^{2+} > Mn^{2+} > Zn^{2+}$.
3) Si un determinado ión se encuentra en entornos de coordinación diferentes, su radio aumentará con el número de coordinación: $4 < 6 < 8$.
4) Si un elemento puede originar iones de carga diferente (números de oxidación), el radio iónico disminuye al aumentar el número de oxidación: $Fe^{2+} > Fe^{3+}$.

Un análisis de los radios iónicos podría explicar la competencia entre el Ca^{2+} y el Cd^{2+} por la calmodulina descrita anteriormente, pues ambos radios iónicos son muy similares (0.99 y 0.97 Å, respectivamente). El mismo razonamiento podría explicar por qué el Cd^{2+} entra a la vacuola de plantas a través del intercambiador de Ca^{2+}/H^+ (Salt y Wagner, 1993). Sin embargo, y de manera opuesta, el K^+ y la Ag^+ poseen radios iónicos comparables (1.33 y 1.26 Å, respectivamente) y su toxicidad no está de ninguna manera relacionada, además de estar agrupados en clases totalmente opuestas de acuerdo con la IUPAC (ver tabla 1.2.2).

Queda claro entonces que la nomenclatura de los metales es compleja y que tanto el término "metal pesado", como la agrupación de metales en clases o la utilización de radios iónicos no refleja fielmente el comportamiento biológico de estos. Sin embargo, la agrupación de metales en clases tendría la ventaja de basarse en propiedades específicas de los elementos, lo cual podría orientar sobre los efectos de un metal, aunque no debe ser considerada como la única herramienta para determinar el mecanismo de toxicidad de un elemento dado.

1.3 *Los metales en la historia del hombre*

Independientemente de su función biológica, los metales también han formado parte importante en la historia del hombre. Periodos enteros de la historia han sido marcados por el descubrimiento y la utilización de un metal o una aleación de metales, como la Edad de Bronce (3500 A.C.) o la Edad del Hierro (1500 A.C.) (Clarkson, 1995). Sin embargo, fue durante la revolución industrial y hasta nuestros días que el uso de metales y metaloides (no metales tales como el As) se volvió indispensable para el desarrollo tecnológico. Por ejemplo, el cadmio y el níquel se usan en la fabricación de baterías, acumuladores, pinturas y diversas aleaciones. El plomo, por mucho tiempo se usó para incrementar el octanaje de las gasolinas y en la fabricación de utensilios de barro vidriado para el consumo de alimentos (Moreno-Sánchez y Devars, 1999). Actualmente se le encuentra en fertilizantes fosfatados y pinturas. El cromo se usa en industrias procesadoras de cemento, curtiduría, material fotográfico, metalurgía y pinturas. El mercurio se usa en la minería, fabricación de equipo eléctrico, amalgamas dentales, herbicidas, industria del papel y celulosa, entre otras (Moreno-Sánchez y Devars, 1999).

De especial mención sería la obtención de plata y oro, de elevado valor comercial y cuya extracción lleva implícita la liberación de otros metales.

Por otro lado, debido al poco cuidado y a la falta de planeación que se tuvo y se tiene actualmente con el manejo de los residuos industriales, la contaminación por metales se ha convertido en un problema de salud pública a nivel mundial. Nriagu y Pacyna (1988) calcularon que la contaminación por "metales pesados" excede la contaminación combinada por desechos radioactivos y orgánicos, con el agravante de que en su calidad de elementos, permanecerán indefinidamente en los sitios contaminados. La limpieza y extracción de los metales de los suelos contaminados es de costo elevado y poco eficiente; por ejemplo, en el año de 1996 el costo variaba entre 50 y 500 dólares americanos por tonelada (Cunningham y Ow, 1996). La limpieza de un acre (1 acre = 0.405 hectáreas) con una profundidad de 3 pies (1 pie = 0.305 m) hubiera tenido un costo de alrededor de un cuarto de millón de dólares. Aparentemente esta es la principal razón por la que suelos contaminados son abandonados (Cunningham y Ow, 1996).

Peor aún es el panorama de las aguas residuales provenientes de las industrias, las cuales se vierten en los ríos u océanos afectando directamente a cuerpos de agua destinados para el consumo humano e intoxicando ecosistemas, los cuales, por magnificación trófica, pueden de igual manera afectar a la población humana (Vázquez-Alarcón et al., 2001).

Una alternativa para substraer metales de suelos y aguas contaminados es mediante el uso de organismos con una elevada capacidad de acumulación de metales. A este proceso se le denomina *biosorción* y *biorremediación* (Salt et al., 1995; Dhankher et a., 2002). Este proceso presenta ventajas sobre los tradicionales métodos fisicoquímicos de extracción en el sentido de costos, uso de recursos renovables y cantidad de material

manejable (Salt et al., 1995; Cunningham y Ow, 1996; Dhankher et a., 2002). De manera natural existen organismos con una elevada capacidad de resistencia y acumulación de metales, a los cuales se les denomina *hiperacumuladores* (Salt et al., 1995). Desafortunadamente, la mayoría de estos *hiperacumuladores* son de lento crecimiento y generan poca biomasa. Para solucionar este problema, una gran cantidad de grupos de investigación se han dedicado, durante las últimas dos décadas, a estudiar y determinar los mecanismos relacionados con la resistencia y la acumulación de metales. La propuesta de estos grupos de investigación, incluido el nuestro, es: después de entender los mecanismos bioquímicos y fisiológicos por los cuales los organismos resisten y acumulan cantidades significativas de metales, será posible manipular (genética o tal vez químicamente) organismos de rápido crecimiento para que adquieran características de organismos *hiperacumuladores.*

La mayor parte de los estudios sobre resistencia a metales se ha llevado a cabo en plantas, con la idea de sanear suelos contaminados (Cunningham y Ow, 1996). Pero la misma idea se aplica a cuerpos de agua mediante el uso de plantas acuáticas u otros organismos acuáticos. De modo que diversos grupos han estudiado la capacidad de bacterias, levaduras, micro- y macro-algas para resistir, unir y acumular diversos "metales pesados" (Trevors et al., 1996; Salt et al., 1995; Navarro et al., 1997; Nedelkoska and Doran, 2000; Hamdy, 2000; Dhankher et a., 2002).

1.4 *El cadmio*

El cadmio es un metal *no esencial* que puede considerarse "metal pesado" (δ = 8.64 g/ml) y agruparse en la clase B (metales suaves), de acuerdo con la clasificación de la IUPAC (Duffus, 2002). En el año 2003, el cadmio fue colocado en el 7º lugar de la lista de

compuestos de alto riesgo de la Agencia de Substancias Tóxicas y Registro de Enfermedades (ATSDR, por sus siglas en Inglés; http://www.atsdr.cdc.gov/clist.html), la cual clasifica a los compuestos con base en su toxicidad, abundancia y riesgo de exposición en humanos. Su uso en la industria es amplio y variado: fabricación de baterías y acumuladores, cables eléctricos, celdas fotoeléctricas, producción de PVC, colorantes, fusibles y joyería, entre otros (Galvão y Corey, 1987). Su liberación al ambiente es a través de los residuos industriales y, de manera muy importante, por la combustión de compuestos fósiles (petróleo, gasolina y diesel; Lagerwerff y Specht, 1970).

Como cualquier metal, los efectos tóxicos del cadmio dependen de la concentración y el tiempo de exposición. De manera general sus efectos están relacionados con la inhibición de enzimas por el bloqueo de tioles esenciales, y con la generación de estrés oxidativo por competencia y liberación de cobre y hierro de sus respectivos sitios dentro de la célula (Guerinot, 2000; Williams et al., 2000; Rea et al., 2004). Tanto el Fe^{3+} como el Cu^{2+} pueden catalizar la autooxidación de compuestos como el NAD(P)H, el ascorbato y tioles orgánicos. El Fe^{2+} y el Cu^{+} pueden, además, participar en la reacción de Fenton, en presencia de peróxido de hidrógeno, generando otras especies reactivas de oxígeno tales como el radical hidroxilo ($HO^{\cdot}$) y el ion hidroxilo (HO^{-}) (Reacción 1 y 2; Hansberg, 2002).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \qquad \text{(Reacción 1)}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + HO^{\cdot} + HO^{-} \qquad \text{(Reacción 2)}$$

El Cd^{2+} también induce peroxidación de lípidos y liberación de compuestos tóxicos como los 4-hidroxialquenales, los cuales son uno de los productos del estrés oxidativo e interfieren con el metabolismo del DNA (Marrs, 1996). A nivel celular, el Cd^{2+} inhibe el

metabolismo energético (glicólisis, fosforilación oxidativa y fotofosforilación) y la duplicación celular (Vallee y Ulmer, 1972; Bonaly et al., 1980; De Filippis et al., 1981; Devars et al., 1998; Mendoza-Cózatl et al., 2002). En mamíferos, el Cd^{2+} se acumula principalmente en el cerebro, hígado y riñón, además de relacionarse con el desarrollo experimental de edema, enfisema y fibrosis pulmonar (Webb, 1972; Sánchez et al., 1994).

En México, la exposición a cadmio se deriva principalmente de la actividad industrial. En San Luis Potosí, una ciudad con una elevada actividad minero-industrial, las mujeres no fumadoras tuvieron hasta 10 veces más Cd^{2+} en la placenta comparado con las mujeres de una ciudad con una actividad minero-industrial baja (Díaz-Barriga et al., 1995). Por otro lado, en un muestreo durante el año de 1996 de las aguas residuales provenientes de la Ciudad de México y usadas para riego de cultivos en el Valle del Mezquital (Hidalgo), el cadmio se encontró por arriba de los límites permisibles de la Norma Oficial Mexicana de aquellos años (0.1 ppm, NOM-002-ECOL-1993; Vázquez-Alarcón et al., 2001). Podemos inferir que las concentraciones en los desagües industriales también excedían por mucho los límites permisibles. Desafortunadamente, esta información no es de libre acceso a la población. Por otro lado, cabe mencionar que la Norma actual contempla mucha mayor tolerancia (y no menor) para las concentraciones de cadmio en aguas destinadas para uso agrícola (0.4 ppm; NOM-002-ECOL-1996). La contaminación por cadmio es un problema de salud pública en México, por lo que es necesaria la investigación dirigida al control y disminución de la concentración de éste y otros metales en aguas destinadas para el riego y el consumo humano. La biorremediación es una tecnología que ofrece una solución directa a este problema de salud pública.

Capitulo 2. Mecanismos celulares de resistencia a Cd^{2+}

En general, los mecanismos de resistencia a Cd^{2+}se pueden clasificar en 5 tipos: (1) unión a la membrana externa o pared celular, (2) cambios en la permeabilidad a iones, (3) expulsión activa del metal, (4) inactivación extra- o intracelular y (5) compartamentalización (Moreno-Sánchez et al., 1999). Para fines de biorremediación, solo son relevantes aquellos mecanismos que estén relacionados con la acumulación intracelular de los metales, por lo que esta tesis se centra en aquellos mecanismos involucrados en la inactivación y compartamentalización intracelular del Cd^{2+}.

El mecanismo más eficiente de resistencia a Cd^{2+} en plantas, levaduras y protistas fotosintéticos es el relacionado con la asimilación de azufre y la síntesis de compuestos tioles. El siguiente trabajo revisa a nivel bioquímico y molecular, el metabolismo relacionado con este mecanismo de resistencia.

pag (14-32 FEMS rev 2005)

ARTICLE IN PRESS

Impact factors of this journal
2004: 8.703 *
* Copyright ISI Journal Citation Report

ELSEVIER

FEMS Microbiology Reviews xxx (2004) xxx-xxx

FEMS MICROBIOLOGY Reviews

www.fems-microbiology.org

Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants

David Mendoza-Cózatl [a,*], Herminia Loza-Tavera [b], Andrea Hernández-Navarro [b], Rafael Moreno-Sánchez [a]

[a] *Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano 1, Col. Sección XVI Tlalpan, 14080 México, D.F. México*
[b] *Departamento de Bioquímica y Biología Molecular de Plantas, Facultad de Química, UNAM, Cd. Universitaria, 04510 México, D.F. México*

Received 24 March 2004; received in revised form 3 August 2004; accepted 17 September 2004

First published online 5 October 2004

Abstract

Glutathione (γ-glu-cys-gly; GSH) is usually present at high concentrations in most living cells, being the major reservoir of non-protein reduced sulfur. Because of its unique redox and nucleophilic properties, GSH serves in bio-reductive reactions as an important line of defense against reactive oxygen species, xenobiotics and heavy metals. GSH is synthesized from its constituent amino acids by two ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. In yeast, these enzymes are found in the cytosol, whereas in plants they are located in the cytosol and chloroplast. In protists, their location is not well established. In turn, the sulfur assimilation pathway, which leads to cysteine biosynthesis, involves high and low affinity sulfate transporters, and the enzymes ATP sulfurylase, APS kinase, PAPS reductase or APS reductase, sulfite reductase, serine acetyl transferase, *O*-acetylserine/*O*-acetylhomoserine sulfhydrylase and, in some organisms, also cystathionine β-synthase and cystathionine γ-lyase. The biochemical and genetic regulation of these pathways is affected by oxidative stress, sulfur deficiency and heavy metal exposure. Cells cope with heavy metal stress using different mechanisms, such as complexation and compartmentation. One of these mechanisms in some yeast, plants and protists is the enhanced synthesis of the heavy metal-chelating molecules GSH and phytochelatins, which are formed from GSH by phytochelatin synthase (PCS) in a heavy metal-dependent reaction; Cd^{2+} is the most potent activator of PCS. In this work, we review the biochemical and genetic mechanisms involved in the regulation of sulfate assimilation-reduction and GSH metabolism when yeast, plants and protists are challenged by Cd^{2+}.

Keywords: Cadmium resistance; Cadmium accumulation; Sulfur assimilation pathway; Phytochelatins; Phytochelatin synthase; γ-Glutamylcysteine synthetase; Glutathione synthetase

Contents

* Corresponding author. Fax: +5255 5573 0926
E-mail address: cozatl@hotmail.com (D. Mendoza-Cózatl)

0168-6445/$22.00
doi:10.1016/j.femsre.2004.09.004

1. Introduction

Some heavy metals such as Cu^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+} are essential in trace amounts for cell metabolism, acting either as enzyme cofactors, mediating redox reactions, and interacting with nucleic acids and proteins [1–3]. Others, such as Cd^{2+}, Pb^{2+}, Hg^{2+} and Ag^{+}, although lacking biological function, enter into the cell through the same transport systems used by essential heavy metals, altering cellular functions [4–6]. A commonly used definition of "heavy metal" is that referring to all chemical elements with a density greater than 5 g ml^{-1}. Metals may also be classified in three types, depending on their reactivity with the functional groups of biomolecules. Class A metals (Al^{3+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, La^{3+}) show more reactivity with oxygen (O > N > S); class B metals (Cu^{1+}, Hg^{2+}, Ag^{1+}) prefer sulfur (S > N > O); and class C metals (Fe^{3+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}) have an intermediate affinity [7].

The toxic effects of heavy metals depend on the time and concentration to which organisms are exposed. Most of the effects are related to their interaction with carboxyl and thiol groups of proteins, to their ionophoretic properties and to their ability to direct or indirectly generate free radicals and hence induce oxidative stress [7–9]. Organisms possess diverse mechanisms to maintain free metal concentrations at levels that do not exceed cellular requirements. These mechanisms include (a) cellular wall binding, (b) changes in ion permeability, (c) active extrusion, (d) bio-transformation, (e) extra- and intracellular chelation, and (f) compartmentation [1,10–14b].

One of the best described mechanisms against heavy metals toxicity in some yeast [15–17], algae [18], photosynthetic protists [19], and plants [12,14a,14b,20] involves their intracellular chelation by either GSH or phytochelatins (PCs), low molecular weight sulfur-containing peptides derived from GSH [12,14a,15, 21a,21b], or both. These peptides may bind a variety of metals in the cytosol and, depending on the organism, the metal-PC or metal-$(GSH)_2$ complexes are actively transported into the vacuole [21a–23]. Like metallothioneins, the PC–metal complexes may activate metal-requiring apo-enzymes; for this reason they have been associated with the regulation of intracellular levels of essential heavy metal and with detoxification of non-essential ones [24,25].

GSH is present in all organisms participating in multiple metabolic processes; for example, intracellular redox state regulation, inactivation of reactive oxygen species (ROS), transport of GSH-conjugated amino acids and other molecules, and storage of sulfur and cysteine affording up to 90% of the non-proteic sulfur in the cell [26,27]. GSH synthesis, starting from inorganic sulfate, requires the sulfur assimilation (SAP) and the cysteine biosynthetic (Cys) pathways (Figs. 1 and 2). The biochemical and genetic regulation of these pathways is complex and is affected by different stress situations such as heavy metal exposure, oxidative stress and sulfur or nitrogen deficiency [28–31]. Several reviews have appeared which analyze the biochemical characteristics of the enzymes and the regulation of the genes involved in the SAP and Cys synthesis in yeast and plants [32–38]. However, none of them have focused on the regulation at the enzymatic and genetic level of cysteine and glutathione synthesis under heavy metal exposure. Therefore, in the present work, we analyze and discuss recent advances in the knowledge of reactions, of enzyme characteristics and properties, and of the biochemical and genetic regulatory mechanisms involved in coping with heavy metal toxicity, specifically with Cd^{2+}. Where appropriate, the lack of information and research on a particular subject is addressed. Biotechnological relevance of this knowledge resides in the possibility of developing organisms with high capacity of Cd^{2+} accumulation for bioremediation purposes.

2. Sulfur assimilation

2.1. Sulfate uptake

Sulfate is co-transported into the cells with $3H^{+}$, in an energy-dependent process catalyzed by specific plasma membrane permeases (Fig. 1, reaction 1) [35,39]. High

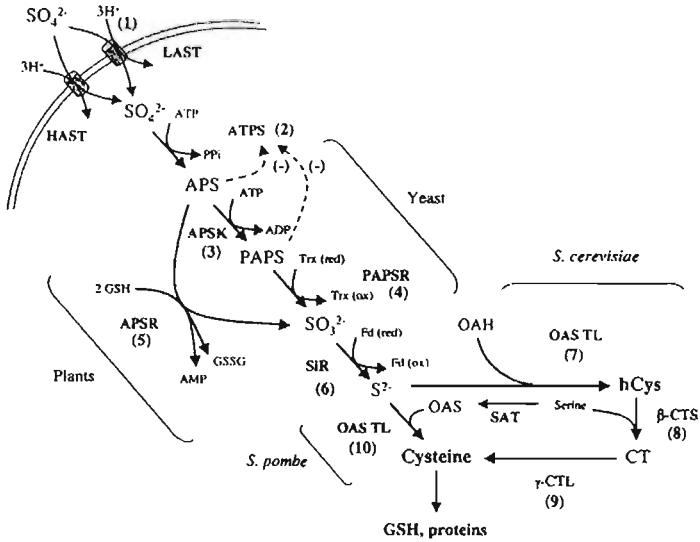


Fig. 1. Sulfate assimilation pathway (SAP) and biosynthesis of cysteine. Numbered reactions are described in the text.

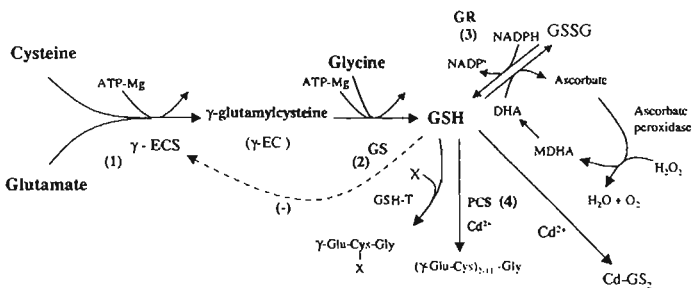


Fig. 2. GSH, PCs synthesis and ROS processing through the ascorbate–glutathione system. AA, amino acid; X, xenobiotic or compound attached to GSH by GSH-*S*-transferases. Numbered reactions are described in the text.

(K_m < 10 μM) and low (K_m > 100 μM) affinity sulfate transporters (HAST and LAST) have been described in different organisms. Although in *Saccharomyces cerevisiae* both activities have been detected (Table 1) [40], only genes encoding HASTs (*SUL1* and *SUL2*) and a third gene (*SUL3*) involved in the transcriptional regulation of *SUL2* [41] have been found. Another gene very similar to *SUL1* and *SUL2* is found in the *S. cerevisiae* genome, but its function is still unknown [42].

In plants, after its uptake by roots, sulfate is distributed into different organs and, to be assimilated, it has to be reduced in a process performed in chloroplasts [36,37]. In these organisms, sulfate transporters are encoded by multigene families, which proteins have not only different sulfate affinity, but also different localization, expression patterns, and regulation [38]. In *Arabidopsis*, HASTs, which are mainly involved in sulfate transport from the environment to root tissues, are encoded by the *Sultr1* family, in which three members have been identified [43–45]. LASTs, expressed in roots and leaves, seem responsible for the sulfate uptake from the apoplast to different tissues inside the plant and are encoded by the *Sultr2* family, with two members [43]. The gene expression of sulfate transporters in plants is also regulated by the availability of sulfate in the external medium, by the sulfate intracellular requirements [41,46] and by GSH [47,48].

In the green algae *Chlamydomonas reinhardtii* two sulfate transporters have been identified, one in cells growing in sulfur sufficient medium and another in sulfur deficient medium [49a]. Analysis of their kinetic parameters suggests that a LAST is present in cells

Table 1
Kinetic parameters for some of the enzymes involved in yeast sulfate uptake and reduction, and Cys biosynthesis

Enzyme	Affinity for different ligands (K_s, K_m or K_i)	V_m	Organism	Reference
LAST, low affinity sulfate transporter	350 μM	7.5 nmol min^{-1}(dry weight)$^{-1}$	*S. cerevisiae*	[40]
HAST, high affinity sulfate transporter	4 μM	7 nmol min^{-1} (dry weight)$^{-1}$	*S. cerevisiae*	[40]
ATPS, ATP sulfurylase	0.17 mM (SO_4^{2-}) 0.07 mM (ATP-Mg) 1 μM (K_i, APS)	2.1 μmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[195]
APSK, APS kinase	n.a.	68 nmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[196]
PAPSR, PAPS reductase	19 μM (PAPS)	7 nmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[197]
SiR, sulfite reductase	30 μM	1 nmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[80]
OAS TL, *O*-acetyl(homo)serinethiol lyase	n.a.	24.8 nmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[127]
CT-βS, CT β-synthase	2.25 mM (hCys) 2.1 mM (serine)	1.2 μmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[198]
CT-γL, CT γ-lyase	0.25 mM (CT)	420 nmol min^{-1} (mg $protein^{-1}$)	*S. cerevisiae*	[199]

The enzymes with the most complete kinetic analysis are shown. n.a., non available.

grown in a complete medium whereas a HAST is induced under sulfur starvation. In the protist *Euglena gracilis*, some activities of the sulfur activating system have been detected in mitochondria [50], but there is no information about the nature of the sulfate transporters in this organelle or in plastids.

Biochemical and genetic characterization of the sulfate transporters of chloroplast membranes has not been addressed either in algae or plants. This is unfortunate since the chloroplast is the major site of sulfate reductive assimilation in these organisms. Several lines of evidence suggest that the sulfate transport system in chloroplasts has different genetic origins and has undergone several changes in the course of the evolution of algae and lower and higher plants. In some algae, such as *Chlorella vulgaris*, *Mososstigma viridae*, *Nephroselmis olivacea*, *and Prototheca wickerhamii*, as well as the primitive liverwort *Marchantia polymorpha*, genes encoding proteins similar to *Cys*T have been identified in the chloroplast genome. *Cys*T is a component of the sulfate permease system in the cyanobacteria *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. PCC6803. In *M. polymorpha* also the *Cys*A gene has been identified. However, the other components, essential for the functioning of the bacterial sulfate permease system, *Cys*W and the sulfate binding protein (*sbp*) have not been found in the chloroplast or nuclear genomes of these organisms (see references cited in [51]). In the unicellular red alga *Cyanidioschizon merolae* the genes *cys*W and *cys*T have been found in the chloroplast genome and the genes *cys*A and *sbp* have been detected in the nuclear genome [52]. In higher plants such as *Arabidopsis* none of the genes encoding a bacterial sulfate transport system have been identified. A nuclear-encoded gene *Sultr4;1* has been suggested to code for a sulfate transporter in the chloroplast [53], localized in the thylakoid membranes [43]. Recently, in *C. reinhardtii* a nuclear-encoded sulfate permease gene (*SulP*), similar to the *Cys*T gene but with a sequence encoding a transit peptide has been identified [51].

2.2. Sulfate activation (ATP sulfurylase)

The second step in the inorganic sulfate assimilation pathway is catalyzed by ATP sulfurylase (ATPS, EC 2.7.7.4; Fig. 1, reaction 2). This enzyme activates SO_4^{-2} via an ATP-dependent reaction that leads to the formation of APS and pyrophosphate (PPi). ATPS catalyzes an energetically unfavorable reaction ($\Delta G^{\circ\prime} = +45.2$ kJ mol^{-1}) that is overcome by metabolic coupling to PPi hydrolysis and APS phosphorylation or reduction in subsequent reactions [54,55]. The product APS is a strong inhibitor of the enzyme ($K_{i_{APS}} = 1$ μM) [56], an apparently common feature in irreversible reactions under physiological conditions [57]. These thermodynamic and kinetic characteristics make the ATPS reaction a likely rate-limiting step of the SAP.

ATPS of *S. cerevisiae* and the filamentous fungus *Penicillium chrisogenum* is an enzyme composed of six identical subunits [58,59]. The kinetic properties of the *S. cerevisiae* enzyme are shown in Table 1. In *P. chrisogenum*, ATPS shows allosteric inhibition by APS and PAPS, which is not observed in the yeast and plant enzymes [59,60]. In plants, the existence of homodimeric cytosolic and chloroplastic isoenzymes of ATPS has been described [61]; the cytosolic isoform seems involved in a pathway non-related to sulfate reduction, but in production of sulfonated compounds [36,62]. The ATPS quaternary structure in protists is unknown, but in *E. gracilis* aplastidic mutants two isoforms localized in cytosol and mitochondria have been identified [63], suggesting that sulfate activation might be performed in both compartments.

Alignment of yeast and plant ATPS gene sequences shows two conserved motifs probably involved in sulfate binding, and a phosphate-binding loop motif, the ATP binding region [64]. In plants, genes encoding cytosolic

and plastidic isoforms have been cloned [61,65–68]. In *S. cerevisiae*, the *MET*3 gene encodes an ATPS [32]. In *C. reinhardtii* the ATS1 gene encodes this enzyme [49b], and in protists no information is available.

2.3. Reduction of sulfate to sulfide (APSK, PAPSR, APSR, SiR)

To accomplish the incorporation of sulfur into biomolecules, specifically amino acids, sulfate in APS is transformed to sulfite and this into sulfide. This process may occur through two different pathways, depending on the organism. One of them involves the phosphorylation of APS by an APS kinase (APSK, EC 2.7.1.25) (Fig. 1, reaction 3) using ATP to produce PAPS and ADP. In the following reaction, PAPS reductase (PAPSR, EC 1.8.99) firstly reacts with reduced thioredoxin and then with PAPS to generate free SO_3^{2-} (Fig. 1, reaction 4). The other pathway involves the direct reduction of APS by APS reductase (**APSR**, EC 1.8.99.x) which uses GSH as an electron source to produce SO_3^{2-} (Fig. 1, reaction 5) [69].

In yeast and many bacteria, SO_3^{2-} is synthesized via APSK [33] whereas in plants, green algae and phototrophic bacteria, sulfate is transformed into sulfite via APSR (for a detailed description of the supporting evidence, see [36,37]).

APSR activity in *Arabidopsis* has not been detected in cytosol [62], and the three genes encoding this enzyme contain transit peptides, indicating that this activity is exclusively localized in chloroplasts [70]. Several plant genes encoding cytosolic and chloroplastic APSKs have been cloned [71]. However, it has become clear that PAPS synthesized by APSK is involved in sulfation of several metabolites such as sulfated flavonols, glucosinolates, steroids and phytosulfokines, but not in sulfate reduction and cysteine biosynthetic pathways [36 and references therein].

In *S. cerevisiae*, *MET14* gene encodes APSK, which has a molecular mass of 23 000 [72], and PAPSR is encoded by *MET16* [73]. A recent phylogenetic study [74] concludes that the presence of an extra iron-sulfur cluster in APSR determines the enzyme specificity and thus separates the APS- and PAPS-dependent sulfate reduction assimilatory pathways. Since PAPS is a highly toxic compound, the cells must strictly regulate its level. In *S. cerevisiae*, a 3′(2′),5′-bisphosphonucleoside-3′(2′)-phosphohydrolase encoded by the *MET22*/*HAL2* gene, has been proposed as the enzyme that transforms PAPS into APS to control its intracellular concentration [32]. In *P. chrysogenum* and *Aspergillus nidulans*, overaccumulation of PAPS seems to be prevented through PAPS-mediated inhibition of ATPS [75].

The previous idea that plants may reduce sulfate through the PAPS pathway has been re-evaluated with experiments with the knockout of the APSR gene in the moss *Physcomitrella patens*. The growth and content of soluble thiol-compounds of this knockout are not affected in a medium with sulfate as the sole sulfur source [76]. However, under Cd^{2+} exposure, growth and content of thiol-compounds of the knockout moss are lower than those of wild type cells. Surprisingly, no PAPSR activity is detected in the APSR knockout moss [76]. These observations suggest that there must be a third pathway for sulfate reduction in *P. patens* which, however, is unable to sustain an adequate cysteine suppiy under Cd^{2+} stress.

A cDNA from the green algae *Enteromorpha intestinalis*, encoding a plastid APSR, has been cloned and the antibodies produced against the recombinant protein cross-reacted with a 45 kDa polypeptide in several chlorophytes but not in chromophytes [77], suggesting that the APSR is not structurally related between these groups. In protists such as *E. gracilis*, it is not clear which sulfate reduction pathway is working. APSK activity has been detected in this protist [78] but whether sulfite is produced from APS or PAPS reduction is still unknown.

Once sulfate has been reduced to sulfite, the subsequent step is identical in bacteria, fungi and plants. Sulfite is reduced to sulfide at the expense of oxidizing three molecules of NADPH, by sulfite reductase (SiR, EC 1.8.7.1; Fig. 1, reaction 6). SiR contains a special acidic heme group called siroheme (redox potential of around −340 mV) and a [4Fe–4S] cluster [79], and catalyzes the reduction of sulfite using electrons donated by ferredoxin. *S. cerevisiae* SiR (see Table 1 for kinetic parameters) is a hetero-tetramer with a MW of 604 kDa and two types of subunits of 116 and 167 kDa [80]. Although yeast SiR is soluble and therefore considered as a cytosolic enzyme, its intracellular location is not well established. Further work by using cell fractionation or immunocytochemistry may show the enzyme localization. Plant SiR has a molecular mass of approximately 65 kDa, is encoded by only one gene, and is exclusively a chloroplastic enzyme [81]. Genes encoding SiR have been isolated from *Z. mays*, *A. thaliana* and *N. tabaccum* [81–83].

2.4. Cysteine biosynthesis (SAT, HAT, OAS/OAH TL, β-CTS and γ-CTL)

Depending on the organism, there are two different ways by which sulfide is incorporated into a carbon backbone to produce cysteine. (1) Sulfide is condensed with *O*-acetylserine (OAS) by OAS thiol lyase (OAS TL), also called OAS sulfhydrylase, to form cysteine directly [33,76] (Fig. 1, reaction 10). In this pathway OAS is synthesized by serine acetyl transferase (SAT). (2) OAS TL also catalyzes the condensation of sulfide with *O*-acetylhomoserine (OAH) to form homocysteine (hCys) (Fig. 1, reaction 7). OAH is synthesized by

homoserine *O*-acetyltransferase (HAT). Then, hCys is transformed into Cys by *trans*-sulfuration (Fig. 1 reactions 8 and 9), i.e. hCys associates with Ser to form cystathionine (CT) by action of cystathionine β-synthase (β-CTS). CT in turn is dissociated into Cys, α-ketobutyrate and ammonia by cystathionine γ-lyase (γ-CTL) [33,34,84,85]. Cys may also be transformed into hCys by reverse *trans*-sulfuration catalyzed by cystathionine γ-synthase and cystathionine β-lyase [32,34]. Kinetic parameters of some of these enzymes are shown in Tables 1 and 2.

The SAT pathway, which is used by enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium* [86], and by plants, has been extensively reviewed [84,87,88]. Fungi use different cysteine biosynthetic pathways depending on the species. *S. cerevisiae* uses the CT pathway [32], whereas *S. pombe* lacks the enzymes for *trans*-sulfuration but has the enzymes for the SAT pathway [34].

Other fungi such as *A. nidulans*, *Neurospora crassa*, *Yarrowia lipolytica* and *Cephalosporium acremonium* synthesize cysteine through both the SAT and *trans*-sulfuration pathways [33]. Similarly, the protist parasite *Trypanosoma cruzi* possesses the enzymes for both sulfur assimilation and *trans*-sulfuration and the genes encoding β-CS and SAT have been cloned [89]. A pathway with parallel reactions that generate a critical metabolite (Cys) ensures its constant supply and implies that the enzymes involved are not rate-limiting steps [57].

In plants, OAS TL and SAT are organized as a bienzyme complex called cysteine synthase. These enzyme activities have been found in cytosol, chloroplasts and mitochondria [90,91], indicating that cysteine may be synthesized in all these compartments (Fig. 1; [84]). The fact that sulfate reduction is only performed in chloroplasts implies that sulfide needs to be translocated from the chloroplast to the other cellular compartments. GSH may mediate such a sulfide exchange since there is a close relationship between the cytosolic and chloroplastic GSH pools [92,93], and probably also the mitochondrial pool.

2.5. Glutathione biosynthesis (γ-ECS, GS)

In contrast to sulfate reduction, GSH biosynthesis is similar in plants, yeast and protists. GSH is synthesized from cysteine in two consecutive ATP-dependent reactions. In the first step γ-glutamylcysteine (γ-EC) is formed from L-glutamate and L-Cys by γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2). The second step is catalyzed by glutathione synthetase (**GS**; EC 6.3.2.3) which adds glycine to the C-terminal of γ-EC forming GSH [26] (Fig. 2, reactions 1 and 2; Table 2). Serine and glycine, required for the synthesis of GSH, derive from 3-phosphoglycerate [94].

Although all γ-ECSs catalyze the same reaction and have similar affinities for their substrates, they are not structurally related among kingdoms [95]. By nucleotide sequence comparison, four types of γ-ECSs have been identified. The first class, the animal γ-ECS, is formed by two different subunits, one catalytic and the other regulatory [96]. The remaining classes are monomeric enzymes. The second class, the bacterial enzyme, shows no identity (8%) with the animal catalytic subunit [95]. The third class comprises the yeast (*S. cerevisiae* and *S. pombe*) and the protist parasite *T. brucei* sequences, with an identity of 40–45% with the animal γ-ECS [97]. The fourth class, represented by the plant enzyme, has only 15–19% identity with the animal catalytic subunit. A conserved motif, a putative GSH binding site, is present in all the species analyzed but the structural differences suggest that they have evolved independently [95].

A functional similarity between enzymes from different kingdoms is that GSH may be a strong competitive inhibitor (K_i, 0.1–20 mM) promoting feedback regulation of the pathway [26], which implies that γ-ECS may be rate-limiting of GSH and PCs

Table 2
Kinetic parameters of the enzymes involved in GSH and PCs biosynthesis, and in Cd^{2+} compartmentation in the vacuole

Enzyme	Affinity for different ligands (K_m)	V_m or k_{cat}	Organism	Reference
γ-ECS, γ-glutamylcysteine synthetase	1.4 mM (Glu) 0.4 mM (Cys) 3.1 mM (K_i, GSH)	13 nmol min^{-1} (mg protein)$^{-1}$	*Candida boidinii*	[200]
GS, glutathione synthetase	0.27 mM (γ-EC) 0.6 mM Gly	170 nmol min^{-1} (mg protein)$^{-1}$	*C. boidinii*	[200]
PCS, phytochelatin synthase	[a,b] 6–13 mM (GSH) [b]9.2 μM ($CdGS_2$) [b]0.54 μM (Cd^{2+})	[a]$k_{cat} = 0.2\ s^{-1}$ [c]0.1 nmol min^{-1} (mg protein)$^{-1}$ (rate determined in cell extracts)	[a]*Silene cucubalus* [b]*A. thaliana* [c]*S. pombe*	[a][113] [b][111] [c][163]
HMT1, heavy metal transporter 1	<30 μM (PC_3)	≈1 nmol min^{-1} (mg protein)$^{-1}$	*S. pombe*	[22]
YCF1, yeast Cd factor 1	39 μM ($Cd\text{-}GS_2$)	15.7 nmol min^{-1} (mg protein)$^{-1}$	*S. cerevisiae*	[158]
Vacuolar Cd^{2+} antiporter	5.5 μM	12.5 nmol min^{-1} (mg protein)$^{-1}$	*S. pombe*	[159]

The enzymes with the most complete kinetic analysis are shown. Superscripts indicate the reference from which the value was obtained.

biosynthesis. Also, L-buthionine-(*SR*)-sulfoximine (BSO) is a specific and potent inhibitor [98], although the bacterial (*E. coli*) enzyme is fully inactivated only after longer periods of incubation than those used for the rat kidney enzyme [99,100]. BSO phosphorylation generates an analog of γ-glutamylphosphate (an intermediate in the catalytic cycle) which binds to the active site producing an irreversible inhibition [98].

GS has been isolated and characterized from different organisms, with their $K_{m_{app}}$ for γ-EC varying between 0.02 and 0.63 mM and the $K_{m_{app}}$ for Gly between 0.3 and 1 mM [26,101] (Table 2). This enzyme is also present in all organisms but similarly to γ-ECS, it differs structurally among kingdoms [102]. Plant GS genes show high identity with the yeast and human sequences but no homology with bacterial genes, suggesting a divergent origin [103]. GSs of plants and mammals are homodimers with a MW of 56–77 kDa per subunit, whereas the *S. pombe* enzyme is a hetero tetramer formed by two subunits of 26 and 33 kDa [104]. GS of the protist parasite *Plasmodium falciparum* is a homodimer, formed by subunits of 77 kDa; its deduced amino acid sequence differs from other GSs particularly at the level of the residues involved in γ-EC binding [103].

2.6. Phytochelatin biosynthesis

Phytochelatins (PCs) are peptides with general formula $(\gamma\text{-Glu-Cys})_{2-11}$-Gly synthesized from GSH by phytochelatin synthase (PCS; Fig. 2, reaction 4). PCs have been detected in some yeast [15–17,105], higher plants [20,106,107], algae [18], and protists [19,108], but not in bryophytes [109,110]. Heavy metals such as Zn^{2+}, Hg^{2+}, Cd^{2+}, Fe^{3+}, Al^{3+}, Cu^{2+} and Pb^{2+} act as enzyme activators, Cd^{2+} being the most potent [12,14a,111].

PCS is a dipeptidyl (instead of a tripeptidyl) transferase that catalyzes PC chain extension in the C- to N-terminal direction, yielding extended (by one γ-EC unit) PC and Gly, GSH or truncated (by one γ-EC unit) PC [111,112]. The PCS reaction involves the Cd^{2+}-independent enzyme acylation in one site by GSH and acylation in a second site by a thiol-Cd^{2+} blocked GSH; these two initial steps generate at least two free Gly molecules. In a third step, the acylated enzyme transfers one γ-EC unit to an upcoming GSH or PC; the other bound γ-EC unit is also released to the medium [111,112] (Fig. 2, reaction 4). PCS is active as a homo-dimer of 41–50 kDa subunits [111,114,115]. Variants of PCs with β-Ala, Ser, Glu or Gln instead of Gly as the ending residue have also been identified in some plants whereas PCs without the C-terminal Gly (desGly PCs) have been found in plants and some yeast [12,106,107,115]. In *Glycine max*, synthesis of homo-PCs, $(\gamma\text{-Glu-Cys})_n$-β-Ala, is catalyzed by a specific homophytochelatin synthase (hPCS) [106]. This enzyme is able to use GSH or homoglutathione (hGSH; γ-Glu-Cys-β-Ala) as substrate; however, synthesis of PCs from GSH as the sole substrate is 5-fold more efficient than hPCs synthesis with a GSH/hGSH mixture [106].

PCS genes have been cloned from *S. pombe*, *A. thaliana*, *Triticum aestivum*, *Brassica juncea*, *Thlaspi caerulescens*, *Glycine max*, *Athyrium yokoscene* and even from the nematode *Caenorhabditis elegans* [106,114,116–119]. PCS deduced sequences from fission yeast and plants have similar N-terminal sequences but a less-conserved C-terminal region [14a,120,121]. These characteristics, together with the observations that a mutation in *A. thaliana* (*cad1-5*) causes premature termination of translation [122] and limited proteolysis of PCS [121] do not prompt total loss of function, have suggested that the N-terminal domain contains the catalytic activity and is presumably highly structured. However, the C-terminal domain also has a role in activity since another *A. thaliana* mutant truncated in this region presented a Cd^{2+}-sensitive phenotype [121,122], and decreased thermal stability and responsiveness to heavy metals [121]. It seems that the less-conserved Cys residues, often presented in pairs, together with some Glu residues present in the C-terminal domain have a role as a binding sensor for heavy metals [14a,120,123].

PCS might be a regulatory enzyme in PCs synthesis since it is the slowest enzyme in the pathway (Table 2) and in *S. pombe* and many adult plants Cd^{2+} exposure does not increase the PCS transcript levels [111,114,117] (see also Sections 4 and 5).

The existence of PCs in *S. cerevisiae* and *Neurospora crassa* has been demonstrated by mass spectrometry [105]. Since only the shortest phytochelatin (PC-2) is detected, and no PCS gene has been found in these fungi, it has been suggested that PCs may be synthesized by a side reaction of GS [124,125] and by carboxypeptidase Y [126]. Detection of PCs when only high pressure liquid chromatography with dithionitrobenzene derivatization is used should be viewed with caution since other compounds such as coumarins may yield a false positive signal [109]. Thus, identification of PCs should be established by applying additional methods such as amino acid sequencing and composition [16–18,20] or mass spectrometry [105,107].

3. Regulation of SAP, Cys and GSH synthesis

3.1. Metabolic regulation

Unicellular and multicellular organisms must be able to maintain a relatively constant intracellular environ-

ment. When mild or severe perturbations in the external medium occur, the organisms adjust their internal functions. There is a range of external changes within which the organisms survive, but a large range in which cellular functions are irreversibly impaired. Thus, there must exist metabolic and genetic control mechanisms that regulate the effect of the external changes over the intracellular environment, allowing the organisms to adapt. The cellular control machinery pursues the modulation (activation or inhibition) of critical enzyme activities through (a) short-term (biochemical) mechanisms consisting of non-covalent interactions with some metabolites and covalent enzyme modification and (b) long-term (genetic) mechanisms consisting in the change of the rates of synthesis and degradation of enzymes. A systematic control analysis of the SAP, Cys, GSH and PCs biosynthetic pathways has yet not been carried out. However, several observations concerning the probably predominant control mechanisms have been reported, which are described below.

In *S. cerevisiae*, sulfate transport is inhibited by internal sulfate and by metabolites derived from sulfate reduction such as APS and cysteine [40]. In addition, OAS increases the activity of all enzymes involved in the SAP [127], whereas cysteine decreases some of them [34,127]. SAT is strongly inhibited by cysteine [127]. In consequence, if cysteine levels diminish, then SAT is activated, increasing the OAS level and activating sulfate assimilation. When cysteine levels are restored, the SAP enzymes and SAT are again inhibited [128].

In *A. thaliana*, the cytosolic SAT form is inbibited by low concentrations of cysteine (2–10 μM), but the mitochondrial and chloroplastic isoforms are insensitive, indicating differences in the regulatory mechanisms of cysteine synthesis among organelles [91]. In watermelon, a single change in the C-terminal region (G277C) of a cytosolic SAT isoform, makes the enzyme cysteine insensitive [129].

For GSH synthesis, it has been assumed that feedback inhibition of γ-ECS by GSH is the prime regulation mechanism [26,101,130]. In turn, different studies have demonstrated that GSH levels change by modifying γ-ECS activity [92,131,132]. Thus, GSH depletion may activate γ-ECS and, hence induce an increase in flux, restoring GSH levels [26,101,133]. It should be noted that GSH must be consumed and not only oxidized to overcome the γ-ECS inhibition and thus activate the pathway. For example, when ROS are elevated the GSH/GSSG ratio decreases, but GSH reductase (GR) is usually able to reestablish this ratio to control values, keeping the pathway unaffected (Fig. 2, reaction 3). Moreover, changes in the GSH/GSSG ratio do not affect the transcription levels of γ-ECS and GS [28]. Glutathione *S*-transferases and PCS (see below) catalyze reactions that consume GSH and in consequence their activation (by increasing availability of their substrates or by overexpression) can overcome the GSH feedback inhibition of γ-ECS resulting in an enhanced pathway flux. Therefore, GSH-consuming enzymes should be also considered when GSH synthesis is analyzed. Cysteine and glycine availability is another mechanism that may contribute to modulate GSH synthesis [29,92,134].

3.2. Genetic regulation

Most of the work addressing the transcriptional regulation of genes encoding enzymes of the SAP, and cysteine, GSH, and PC synthesis has focused to the understanding of gene activity under sulfur starvation. However, the response of these genes to Cd^{2+} exposure has not been fully addressed. In some organisms and depending on the Cd^{2+} concentration tested, the cysteine and GSH levels may diminish after the first minutes of Cd^{2+} exposure but hour or days later these levels are restored or, in some cases, enhanced over basal values, suggesting transcriptional activation of SAP and GSH synthesis by Cd^{2+} [19,28,29,31,110,135]. Whether this response is directly related to Cd^{2+} or to GSH or cysteine depletion induced by Cd^{2+} stress has not been analyzed. In general, all the genes encoding enzymes of these pathways are transcriptionally up-regulated by sulfur starvation and, in the cases where Cd^{2+} response has been analyzed, most of them also respond by increasing transcriptional activity.

In *S. cerevisiae*, *SUL1 and SUL2* genes increase their mRNA levels by 9–14 and 5 times, respectively, after Cd^{2+} exposure [136,137]. Genes encoding HASTs in plants increase their mRNA levels in response to low sulfate availability [35,46,48], and to the internal increase in OAS [138,139]. Their transcript levels diminish with addition of sulfate, cysteine or GSH to the culture media [47,48,139]. In Cd^{2+}-exposed *B. juncea* roots, but not in leaves, a decrease in a putative low-affinity transporter transcript is observed, suggesting a tissue-specific regulation [140]. In *Z. mays*, Cd^{2+} exposure induces an increase in the expression of a HAST gene in roots, which correlates with an enhanced uptake of sulfate [141]. More studies on gene expression and kinetics are required to establish whether sulfate transporters are Cd^{2+} responsive, particularly in Cd^{2+} resistant organisms.

Expression of ATPS genes is stimulated by sulfate starvation [31]. Cd^{2+} exposure induces an increase in the *A. thaliana APS3* (ATPS) transcript level (13-fold) [31], in roots and leaves of *B. juncea* [140], and in the *MET3* gene (ATPS) of *S. cerevisiae* [137,140]. Cd^{2+} stress also promotes a 6-fold increase in the ATPS protein level in *S. cerevisiae* [142], although activity was not determined.

Analysis of *S. cerevisiae* gene expression in response to 0.03 mM $CdCl_2$, using microarray analysis, determined that *MET14* (APSK) and *MET16* (PAPSR) increased their expression by 21- and 6-fold, respec-

tively, over the control [136]. The proteome analysis of *S. cerevisiae* shows that the PAPSR protein increases 5-fold in response to 1 mM Cd^{2+} exposure [142]. In *A. thaliana*, APSR transcription increases when the plant grows under sulfur starvation [70,77]. Genes encoding APSR in *B. juncea* and *A. thaliana* (*PRH19* and *PRH43*) are also transcriptionally up-regulated by Cd^{2+} stress [31,140].

In the proteome analysis of the *S. cerevisiae* Cd^{2+}-response, the SiR β subunit increases 2.5 times [142], while in the transcriptome analysis, *MET10* (encoding the β subunit) increases its amount of mRNA by 5.5 times [136]. In the presence of 0.2 mM $CdCl_2$ the *sir* transcript levels increase up to 2-fold [31].

In *A. thaliana* the OAS TL cytosolic gene *Atcys-3A* is transcriptionally up-regulated by Cd^{2+} exposure bringing about an increase in GSH synthesis [29]. In *S. cerevisiae*, the expression of the CT-γ-lyase gene is increased by 13 times after exposure to 1 mM $CdCl_2$ for 1 h [137]. In protists, there is no information about the SAP genes response to Cd^{2+} exposure.

The *S. cerevisiae GSH1* (γ-ECS) gene is transcriptionally up-regulated by Cd^{2+} [143] whereas the protein shows a stimulation index of 10 in response to Cd^{2+} stress [142]. Using the *S. cerevisiae* γ-ECS promoter sequence, a 20-fold increase in the associated reporter gene activity was found after its exposure to 0.1 mM $CdCl_2$ [144]. In contrast, Cd^{2+} does not affect the γ-ECS gene in *S. pombe* [145]. In plants, Cd^{2+} induces an increase in the γ-ECS mRNA [28,29,146]. Post-translational regulation, such as phosphorylation/dephosphorylation, has been invoked to explain the rapid increase in γ-ECS activity after Cd^{2+} exposure observed in *A. thaliana*, without an increase in γ-ECS transcript levels [147]. However, to date no experimental evidence of covalent modulation of γ-ECS has been described.

After Cd^{2+} exposure, the levels of GS transcripts increase around 2-fold in *A. thaliana* [28,31]. These increments are not as strong as those observed for other genes encoding enzymes of the SAP [31]. In *S. pombe*, Cd^{2+} does not alter the transcription of the GS gene [145]. In protists, there are no reports about transcriptional regulation of γ-ECS or GS induced by Cd^{2+} exposure.

PCS is transcriptionally up-regulated (2-fold) after Cd^{2+} exposure in 5 day-old seedlings of *A. thaliana* and in *T. aestivum* roots [148,116]. In *B. juncea* leaves, but not in roots, a 4-fold increase in the PCS content was found after Cd^{2+} exposure, without a concomitant increase in mRNA [117], whereas in *S. pombe* and mature *A. thaliana* plants Cd^{2+} exposure does not affect the transcript level of PCS [111,114].

Genes encoding SAP enzymes are coordinately regulated in *S. cerevisiae*. Several regulatory molecules have been implicated in the modulation of the gene expression. AdoMet (*S*-adenosyl-methionine) was first proposed as the main co-repressor [32]. However, recent data sustain the idea that cysteine is the pathway specific co-repressor for the genes encoding SAP enzymes while OAS is the main co-activator [33,127,149]. AdoMet seems to act as a co-factor in the cysteine-mediated repression [149]. Likewise, GSH has a moderate repressive role on specific genes such as *MET25* encoding OAS TL, which synthesizes homocysteine, the precursor of cysteine in *S. cerevisiae* [33]. Based on the finding that cysteine biosynthesis in this yeast is performed by the CT pathway instead of the OAS pathway, the role of OAS in this organism seems to be only as a regulatory element and not as a metabolic intermediate [33].

In plants, an integrative metabolic regulation view emerges from the observation that OAS induces the expression of HAST genes in barley and *A. thaliana*. OAS also differentially mediates the expression of organ specific isoforms of ATPS, APSR, OAS TL, and γ-ECS in *A. thaliana* [30,138,139]. In turn, cysteine and GSH are known to be negative regulators of gene expression of sulfate transporters, ATPS and APSR [150–152]. Thus, OAS, cysteine and GSH levels establish a connection between enzyme activities, metabolic flux and gene expression.

The transcriptional factors involved in the regulation of the genes that respond to Cd^{2+} stress have not been identified in plants and protists. In *S. cerevisiae*, the induction of the genes encoding the enzymes of the SAP and GSH biosynthesis by Cd^{2+} depends upon the transcription factor, Met4p, which is recruited by Met31p, Met32p and Cbf1p, to form a transcriptional complex involved in the activation of most of the methionine biosynthetic genes [153–155]. The Yap1 factor, which mediates the oxidative stress response, is also involved in the Cd^{2+} response [142,154]. Mutants that do not express this factor (Yap1Δ) are hypersensitive, whereas strains overexpressing the factor are hyper-resistant to Cd^{2+} [156,157]. Given the importance that the detoxification systems play in the Cd^{2+} response, it has been suggested that the YCF1 and *gsh1* genes, which encode proteins responsible for sequestration of Cd^{2+} in the vacuole (see below), are the primary targets by which Yap1 exerts a control of Cd^{2+} resistance [142]. The Skn7 transcription factor, which cooperates with Yap1 to activate the hydrogen peroxide response, appears to negatively act in Cd^{2+} response, as a repressor of several genes [156].

Functional proteomic studies in *S. cerevisiae* show that many proteins change in response to Cd^{2+}, particularly enzymes of the SAP and heat shock proteins. Some others are oxidative stress enzymes (catalase T, thioredoxin, thioperoxidase, Mn-superoxide dismutase, Cu/Zn superoxide dismutase, alkylhydroperoxide reductase), proteases and enzymes from carbohydrate metabolism not related with stress responses [137,142]. These latter enzymes are isoforms with lower sulfur content that are synthesized to replace existing enzymes. In control conditions the amount of sulfate incorporated

into these proteins is 79% but diminishes to 19% under Cd^{2+} stress. Under this condition, 70% of sulfate is incorporated into metabolites of the GSH biosynthesis (CT, γ-EC and GSH). Thus, the sulfur amino acids may be directed to the massive production of GSH. The fact that the "new enzymes" have less sulfur amino acids, also makes them less susceptible to the deleterious Cd^{2+} effect since Cd^{2+} will bind them with a much lower affinity. Based on the linear correlation found between the induction factor of transcript levels and the induction factor of protein content, it was concluded [137] that the yeast Cd^{2+} response is essentially regulated at the transcriptional level. However, this might be an overinterpretation since no determination of enzyme activity, flux, and time-dependent metabolite variations of the SAP, Cys and GSH pathways were made.

4. Mechanisms of GSH- and PC-mediated Cd^{2+} resistance

4.1. Yeast and plants

Compartmentation in the vacuole appears to be the most important mechanism for Cd^{2+} resistance in *S. cerevisiae*, *S. pombe*, *Candida glabrata*, and plants [12,17,22,23,158,159]. Cadmium can be transported into the vacuole as a free ion or associated with thiol-compounds (GSH or PCs) [22,23,158,159]. In *S. pombe*, *C. glabrata*, some algae and plants, PC–Cd complexes, free Cd^{2+} and sulfide form high molecular weight (HMW) complexes inside the vacuole which are the ultimate and more stable storage of Cd^{2+} inside the cell (Fig. 3(a)) [17,160–163]. It should be noted that the available K_m values of the transport systems for free Cd^{2+} (see Table 2) are relatively high (>30 μM), whereas the K_d value of PCS for free Cd^{2+} is low (0.54 μM). Although no data on free Cd^{2+} concentrations have been described in organisms exposed to toxic $CdCl_2$ concentrations, the K_m and K_d values for Cd^{2+} may be used as a reference for the expected range of free Cd^{2+} concentrations that can be reached in the cytosol to turn on the cellular Cd^{2+} response.

In *S. pombe*, PCs are transported into the vacuole by an ABC-type transporter, encoded by the *hmt1* gene [22,164] (heavy metal tolerance 1; Fig. 3(a), reaction 1). The HMT1 protein is related to multi-drug resistance proteins (MRPs), which catalyze the ATP-driven transport of GSH S-conjugates for xenobiotic detoxification. However, unlike MRPs, HMT1 has only one transmembrane and one nucleotide-binding domain [164]. HMT1 activity is sensitive to vanadate, but not to inhibitors affecting the vacuolar H^+-ATPase or to ionophores that abolishes the pH gradient across the vacuolar membrane [22], indicating a strict dependence on ATP hydrolysis, but not on a H^+ gradient, for driving uptake of Cd^{2+}-PCs complexes.

HMT1 is specific for PC transport and *hmt1*$^-$ cells are Cd^{2+} sensitive, which indicates that the resistance mechanism in *S. pombe* depends on the proper storage of PCs in vacuoles. HMT1 gene expression is not Cd^{2+} inducible, although its overexpression enhances Cd^{2+} accumulation and resistance [22].

In *S. cerevisiae*, Cd^{2+} is also stored into the vacuole as a complex, but in contrast to *S. pombe* and plants, it is

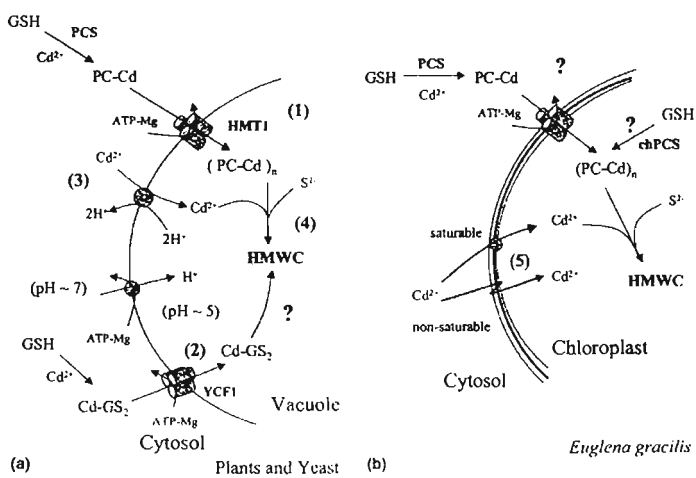


Fig. 3. Mechanism of Cd^{2+} resistance in plants, yeast and protists. (a) Cd–PC complexes are compartmentalised into vacuoles or tonoplasts. In yeast, HMT1 is the protein responsible for transport of the Cd–PC complex [22]. In plants, a similar activity has been described but no homologous gene has yet been found [23,167]. (b) Proposed mechanism for Cd^{2+} resistance in *E. gracilis*, in which most of the Cd^{2+} and PCs are located inside the chloroplast [19]. Numbered reactions are described in the text.

transported as Cd-bis(glutathionate) (Cd–GS_2) by YCF1 (yeast cadmium factor) [158,165] (Fig. 3(a), reaction 2). YCF1 shares amino acid sequence similarity with MRPs and HMT1, its reaction is ATP-dependent and uncoupler-insensitive, but in contrast to *hmt1*, *ycf1* expression is up-regulated when cells are exposed to Cd^{2+} [158]. YCF1 has a $K_{mCd-GS2}$ of 39 μM and V_m of 15.7 nmol min^{-1} (mg protein)$^{-1}$ [134]. YCF1 may transport Cd–GS_2 and some GSH-conjugates such as dinitrophenyl-GSH but no PCs [158]. HMT1 may transport PCs and Cd–PC complexes but apparently no other GSH-conjugates [22]. Recently, another MRP named BPT1 (from bile pigment transporter) has also been related to Cd^{2+} resistance in *S. cerevisiae* [166], although its activity does not replace YCF1 function. In plants, PCs are also transported to the tonoplast in an ATP-driven process similar to that reported in *S. pombe* [23]. However, no *hmt1* or *ycf1* homolog genes have yet been identified [167].

Other vacuolar membrane transporters in *S. pombe*, *S. cerevisiae* and in plants can also take up free Cd^{2+}. Uptake of Cd^{2+} is not affected by vanadate but it is inhibited by NO_3^- and does not occur when a non-hydrolyzable ATP analog (AMP-PNP) is used. This suggests that Cd^{2+} transport is driven by the Δ pH generated by the vacuolar H^+-ATPase [159,168] (Fig. 3(a), reaction 3). In *Avena sativa* vacuoles, the nigericin-dependent Cd^{2+} uptake has a K_{mCd} of 5.5 μM and V_m of 12 nmol min^{-1} (mg protein)$^{-1}$, whereas in *S. pombe* vacuoles, the initial Cd^{2+} uptake rate with 8 μM $CdCl_2$ is 37 nmol min^{-1} (mg protein)$^{-1}$ [22,159]. It is not known whether in the cytosol of Cd^{2+}-exposed cells, the free Cd^{2+} concentration may reach values of 5–8 μM or higher. Due to the tight Cd^{2+} binding to a variety of biomolecules, including thiols, it is likely that the free Cd^{2+} concentration be lower than 10 μM under exposure to non-toxic $CdCl_2$ concentrations. A non-manageable level of intracellular free Cd^{2+} is reached when the cellular chelating capacity is surpassed, which may bring about the usually observed deleterious effects of Cd^{2+} toxicity on cell physiology.

Five protein families have been implicated in the transport of Cd^{2+} through cell membranes: (1) cation/H^+ antiporter family; (2) CPx-type ATPases; (3) Nramp, natural resistance-associated macrophage proteins; (4) CDF, cation diffusion facilitator family, also named cation-efflux family; (5) ZIP, ZRT-IRT-like proteins (for a detailed description of these families, see [169–171]).

In *S. cerevisiae*, Nramp, CDF and ZIP proteins are responsible for Cd^{2+} trafficking between cytosol and vacuole. SMF1, one of the three Nramp members identified in *S. cerevisiae*, is able to take up Cd^{2+}, Cu^{2+} and Mn^{2+} [172]. SMF1 is located in the vacuole, although when cells are grown in the absence of heavy metals, it may also be located in the plasma membrane [173].

The *S. cerevisiae* ZRC1 and COT1 proteins, members of the CDF family, have been related to detoxification of heavy metals [168,174–177]. Both proteins are responsible for Zn^{2+} storage in the vacuole, but at Zn^{2+}-limiting conditions, ZRC1 is more important than COT1 for metal storage [177]. Cells deleted for either or both genes encoding these proteins show a decreased resistance to Co^{2+}, Zn^{2+} and Cd^{2+}, with the *cot1* deletion being more detrimental to Cd^{2+} exposure than the *zrc1* deletion [177,178]. These observations suggest that both proteins are involved in the uptake of Cd^{2+} into the vacuole and that they have different affinities for heavy metals or have different expression in response to heavy metal exposure.

The *S. pombe* genome has revealed three CDF sequences, and the deletion of one of them (Δ*SpZRC1*) also results in extremely high Zn^{2+} and Co^{2+} sensitivity [178]. The only ZIP family member found in *S. cerevisiae* (ZRT3) seems to play a role different to heavy metal detoxification since it releases Zn^{2+} from the vacuole; ZRT3 plays a crucial role when cells are grown in Zn^{2+}-limited medium [176]. All the transporters described above are transcriptionally regulated by Zn^{2+} availability in the growth medium [176,177]. Overexpression of ZRC1 and COT1 increases Zn^{2+} resistance, whereas ZRT3 overexpression results in poor growth [174–176]. Unfortunately, neither the response of these transporters to Cd^{2+} exposure nor the effect of their overexpression on Cd^{2+} resistance has yet been explored.

In plants, proteins of the cation/H^+ antiporter, ZIP, Nramp and CDF families transport Cd^{2+} (see 171 and references therein). To date, only CAX2 (a cation/H^+ antiporter) from *N. tabacum* has proven to be located in vacuole membranes [179]. CAX2 is not up regulated by $CdCl_2$ exposure, but heterologous expression of *A. thaliana* CAX2 in tobacco plants leads to 15% more Cd^{2+} accumulation than that attained in control plants and to a 1.6- to 3-times increase in the Cd^{2+}, Mn^{2+} and Ca^{2+} uptake in isolated tonoplast vesicles [179].

In summary, Cd^{2+} can enter vacuoles in two ways: as a free ion, or complexed with GSH or PCs. In *S. pombe*, *C. glabrata* and plants, Cd^{2+}–PC complexes incorporate sulfide to form HMW complexes (Fig. 3(a), reaction 4) around a CdS crystallite core [16,17,160–163]. The vacuolar S^{2-}/Cd^{2+} ratio varies between 0.16 and 2 [17,160,180]. The addition of sulfide confers a higher Cd^{2+}-binding capacity and enhanced stability to the complex, bringing about full inactivation of the toxic Cd^{2+} ion. Impairment in any of these processes, *i.e.* sequestration, transport, and HMW complex formation, results in a Cd^{2+}-hypersensitive phenotype [160,161, 164].

Studies in *C. glabrata* have shown that the molecular weight and peptide composition of HMW complexes may vary depending on the sulfide content and the growth media composition [17,181]. Cells grown in synthetic complete medium form HMW complexes lower

than 50 kDa whereas additional sulfur supply at the late logarithmic phase of culture (from 0.2 to 2.1 mM of methionine) gives rise to higher than 50 kDa complexes [181]. In addition, *C. glabrata* cultured in rich medium contains the usual CdS cores coated with GSH and γ-EC, whereas when it is cultured in minimal medium the CdS core is coated by PC_2 and desGly PC_2 [182]. The factor involved in this difference is not known; however, it is clear that γ-EC/GSH coated complexes are less stable than those formed by PCs [182].

HMW complexes can be formed in vitro by mixing sulfide, Cd and PCs [165]. However in *S. pombe*, two purine biosynthetic enzymes, adenylosuccinate synthetase and succinoaminoimidazole carboximide synthetase, are required for HMW complex formation [160,161]. It is suggested that these enzymes use a sulfur analog of aspartate, cysteine sulfinate, to produce intermediates or carriers in sulfate assimilation to form HMW complexes. However, the reaction sequence in vivo has not been fully elucidated [161].

4.2. *Euglena* and unicellular green algae

Euglena gracilis is a photosynthetic protist with high Cd^{2+} tolerance and high Cd^{2+} accumulating capacity, in which the effects of Cd^{2+} exposure have been more extensively studied. Probably due to the absence of a specialized reservoir organelle such as a plant-like vacuole, in Cd^{2+} exposed light-grown cells more than 60% of the accumulated Cd^{2+} resides inside the chloroplast [19], whereas in dark-grown cells most of it is located in mitochondria [108].

Several reports have shown that the SAP, including GSH and PCs synthesis, is related with the Cd^{2+} resistance mechanism in this protist [18,19,108,183]. When photosynthetic *E. gracilis* cells are exposed to 0.2 mM $CdCl_2$, their cellular levels of cysteine, GSH and PCs increase, being 12-fold larger than in non-exposed cells. These metabolite variations occur in the cytosol and more remarkably in the chloroplast [19].

The mechanism of how PCs are synthesized and stored in *E. gracilis* is under investigation in our laboratory. In this protist, PCs are found in the cytosol, chloroplasts and even mitochondria after Cd^{2+} exposure [19,108]. These findings may be explained by either of the following mechanisms. (1) PCs are synthesized in the cytosol where they sequester Cd^{2+}; the Cd–PC complexes are subsequently transported into the chloroplast and mitochondria. (2) PCs are synthesized inside the organelles where they bind Cd^{2+}, which is transported as a free ion, and form HMW complexes. (3) Both processes co-exist and PCs are synthesized in the three cellular compartments (Fig. 3(b)).

The first possibility implies the existence of an ABC-like transporter to mobilize Cd–PC or $Cd–GS_2$ into the mitochondria and chloroplasts of *E. gracilis*, similar to that present in *S. pombe* and plant vacuoles; however, this kind of transporter has not yet been described for chloroplasts or mitochondria.

The second possibility implies the presence of a free Cd^{2+} transporter in the organellar membranes and a PCS activity inside the organelles. Indeed, *E. gracilis* chloroplasts are able to take up Cd^{2+} in its free form. This process involves at least two components [19], one saturable (V_m = 11 nmol min^{-1} mg $protein^{-1}$ and K_m = 15 μM) and a non-saturable one (0.12 nmol min^{-1} mg $protein^{-1}$ μM^{-1}) (Fig. 3(b), reaction 5). In addition, net PCs synthesis by Percoll-purified chloroplasts incubated with 0.5 mM $CdCl_2$ (0.15 nmol h^{-1} mg $protein^{-1}$) suggests that a PCS isoform is localized inside *E. gracilis* chloroplasts. Moreover, we have detected HMW complexes in chloroplasts isolated from Cd^{2+}-exposed *Euglena* (Mendoza-Cózatl D, Rangel-González E, Moreno-Sánchez R, unpublished data). These observations support the existence of the second mechanism in *Euglena* chloroplasts, but they do not exclude the existence of the first possibility.

It is difficult to understand how a Cd^{2+} resistance mechanism might be biochemically and structurally supported in either chloroplasts or mitochondria, which perform several essential reactions and provide energy for the cell function. Hence, Cd^{2+} accumulation inside these cellular compartments might be a consequence of the organelle characteristics in this particular protist rather than a specific mechanism for Cd^{2+} resistance. Cd^{2+} enters the chloroplasts, probably using the transport systems for essential ions. Thus, it seems likely that in the chloroplast mechanisms to maintain low levels of internal free ion metals (essential and non-essential) have evolved to prevent their toxic effects.

PCs have been described in several groups of algae including chlorophytes, xanthophytes, diatoms, phaeophytes and rhodophytes [18]; but there is no information about the detailed function of these peptides or whether they are involved in Cd^{2+} transport to intracellular organelles. In addition to *E. gracilis*, it has been found that 60% of accumulated Cd^{2+} resides inside the chloroplast in a cell-wall deficient strain of *C. reinhardtii* [184]. HMW complexes are also found in this organelle [184], but the origin of plastid PCs and the mechanism by which Cd^{2+} is transported into the chloroplast of *Chlamydomonas* is still unknown. *Chlorella* synthesizes PCs and forms HMW complexes in response to Cd^{2+} exposure, although the intracellular localization of these compounds is also unknown [11,18]. However, it has been established for *Chlorella* that the cell wall represents the first line of defense against Cd^{2+} exposure. In fact, at 100 μM $CdCl_2$, 50% of the cellular Cd^{2+} is bound to the cell wall [185].

Increased PCs synthesis has also been associated with higher resistance to oxidative stress. In the green algae *Dunaliella tertiolecta*, an increase in PCs induced by

Zn^{2+} exposure, results in an enhanced resistance to ROS caused by H_2O_2 and paraquat [186]. This protection was the result of a stronger reaction of H_2O_2 with PC_3 than with GSH or ascorbate. Pre-treatment of *D. tertiolecta* with Zn^{2+} brings about an increased resistance to Cd^{2+}, through a PC-mediated process [186].

Chlorella vulgaris and some plants accumulate proline when exposed to heavy metals [187,188]. Because proline may directly react with free radicals diminishing the damage by oxidative stress [189], it was proposed that organisms with high levels of proline might show a higher Cd^{2+} resistance than organisms with low proline levels [188]. This hypothesis has been tested by using transgenic *C. reinhardtii* cells overexpressing Δ^1-pyrroline-5-carboxylate synthetase, the enzyme that catalyzes the first step in proline biosynthesis in plants. The transgenic cells contained 80% more proline than non-transformed cells. Under Cd^{2+} exposure, transformed cells showed a more vigorous growth, 4.1 times more Cd^{2+} accumulation, 80% more GSH, apparently more PCs and a decrease in the GSSG levels. GSH contends simultaneously with Cd^{2+} detoxification and oxidative stress induced by Cd^{2+}. Therefore, it appears that in the presence of high proline, GSH availability for PCs synthesis is elevated, resulting in a more Cd^{2+} resistant organism [188].

5. Enhancing glutathione and phytochelatin synthesis

It is desirable that organisms designed for bioremediation, or for enhancing the content of essential metals in food crops, must have some, if not all, of the following characteristics. (1) High heavy metal uptake rate; (2) an efficient mechanism for metal sequestration-inactivation; (3) appropriate heavy metal storage; (4) large biomass production; and (5) in the case of plants, an adequate root-to-shoot transport of the metal. The first point, related to the uptake of the heavy metal from the environment, is probably the process for which modification may be more critical due to the dependence of the subsequent processes on this initial event. In the last decade, the cloning of the genes involved in the SAP, GSH-PCs synthesis, and in intracellular heavy metal and Cd-complex transport has prompted several groups to improve the heavy metal resistance-accumulation capacity of the cells by overexpressing some of the enzymes involved in such processes.

Although some groups have obtained promising results regarding enhanced GSH biosynthesis and Cd^{2+} resistance [92,190–193], others have not succeeded [92,132,191–194]. This is probably because most of the overexpression experiments have increased the amount of only one enzyme, the putative rate-limiting step, without considering substrate availability [194] or product inhibition for the overexpressed enzyme or the side accumulation of toxic intermediaries [132]. Moreover, there are no experimental data on how GSH and PC synthesizing pathways may respond to an increased demand of cysteine and GSH (for instance under Cd^{2+} stress). Thus, it is not known up to which limit an enzyme activity may be elevated without (a) compromising the cysteine and GSH pools (which are also used for protein synthesis, ROS and electrophilic compounds processing) or (b) provoking the accumulation of reactive intermediaries such as oxidized γ-EC (ESSE) [132]. Furthermore, as discussed throughout this review, heavy metal resistance and accumulation is not related to only one enzyme activity, but it is the result of a complex regulation at the genetic and enzymatic levels of several simultaneous processes (sequestration, transport and storage).

We are aware that simultaneous overexpression of several enzymes may be technically difficult. Then, an appropriate analysis of control of Cys, GSH and PCs synthesis, in both unstressed conditions and during Cd^{2+} exposure, may lead to the identification of a minimal set of enzymes that need to be modulated for reaching enhanced fluxes or metabolite concentrations. Such a set of enzymes to be modified probably may vary between organisms. However, analysis of the kinetic properties of the enzymes of the yeast pathway (Tables 1 and 2), but also of the plant pathway (data not shown), suggests that γ-ECS and PCS may be the most relevant steps in the control of flux, with ATPS and the Cd–PC (or $Cd–GS_2$) vacuole transporter playing a secondary but still significant role. Modulation of flux towards PCs might also be exerted downstream in the pathway, i.e., sulfide transport and incorporation into HMW complexes but this has not yet been explored.

PCS meets the requirements for an ideal target to be genetically manipulated. (1) It is a homodimeric enzyme coded by one gene; (2) its product is directly involved in sequestration and transport of Cd^{2+}; and (3) different PCS sequences from several organisms are now available. However, some confounding results raise doubts about PCS as a genetic target. For instance, in *A. thaliana* PCS overexpression causes Cd^{2+} hypersensitivity [194]. High expression of *C. elegans* PCS in a Cd^{2+} hypersensitive strain of *S. pombe* (Sp27) is less effective in rescuing the Cd^{2+} hypersensitivity than low expression of the enzyme [119]. In the case of *A. thaliana*, the possibility of a limited GSH supply is apparently excluded since the total glutathione content (GSSG + GSH) is the same in control and transformed plants. However, Cd^{2+} exposure may alter the GSH/GSSG balance and this ratio was not evaluated. Therefore, GSH availability may be partially responsible for the confounding result.

On the other hand, the most successful strategy to increase the GSH content has been the overexpression of γ-ECS [92,190–192]. However, an overwhelming activity of γ-ECS over GS activity may also cause oxidative stress by accumulation of ESSE [132]. Therefore, an

adequate increase in PCs synthesis together with a sufficient supply of GSH without exceeding the PCs transport rate nor the GS activity may bring about improved results than those obtained by unrestricted overexpression of just one enzyme. Future work is required to determine the control of cysteine, GSH and PCs synthesis and to establish which set of enzymes need to be modulated to obtain enhanced fluxes and increased metabolite concentrations with a minimum of side-effects.

6. Concluding remarks

1. A widespread response to Cd^{2+} stress in yeast, plants and protists is the enhancement in the rate of sulfur assimilation, cysteine, GSH, and in some organisms, PCs synthesis through enzymatic activation and up-regulation of some genes involved in these biosynthetic pathways.
2. Compartmentation of Cd–PC complexes is not restricted to vacuoles or tonoplasts. In photosynthetic protists and some unicellular green algae, other organelles such as chloroplast or mitochondria may be involved in the Cd^{2+} resistance mechanism.
3. If Cd^{2+} resistance is associated with its intracellular accumulation, then the organism with such abilities may be used in the bioremediation of heavy metal-polluted soils and water bodies.
4. A systematic study of the control of cysteine, GSH and PCs synthesis is required to elucidate the set of enzymes to be enhanced for improving the Cd^{2+} resistance-accumulation ability of any given organism. This set of enzymes may vary between species.

Acknowledgement

The present work was partially supported by Grant CONACyT-México No. 43811-Q.

References

[1] Kägi, J.H.R. and Schäffer, A. (1988) Biochemistry of metallothioneins. Biochemistry 27, 8509–8515.
[2] Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L. and Eide, D. (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. Proc. Natl. Acad. Sci. USA 95, 7220–7224.
[3] Clarkson, T. (1995) Health effects of metals: a role for evolution. Environ. Health Perspect. 103, 9–12.
[4] Paulsen, L.T. and Saier, M.H. (1997) A novel family of ubiquitous heavy metal ion transport family. J. Membr. Biol. 156, 99–103.
[5] Thomine, S., Wang, R., Ward, J.M., Crawford, N.M. and Schroeder, J.I. (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. Proc. Natl. Acad. Sci. USA 97, 4991–4996.
[6] Rogers, E.E., Eide, D.J. and Guerinot, M.L. (2000) Altered selectivity in an *Arabidopsis* metal transporter. Proc. Natl. Acad. Sci. USA 97, 12356–12360.
[7] Nieboer, E. and Richardson, D.H.S. (1980) The replacement of the non descript term "heavy metal" by biologically and chemically significant classification of metal ions. Environ. Pollut. 1, 3–8.
[8] Valle, B.L. and Ulmer, D.D. (1972) Biochemical effects of mercury, cadmium, and lead. Annu. Rev. Biochem. 41, 91–128.
[9] Kamiski, L.P. (1992) Hg^{2+} and Cu^{+} are ionophores, mediating Cl^{-}/OH^{-} exchange in liposomes and rabbit renal brush border membranes. J. Biol. Chem. 267, 12218–12250.
[10] Reed, R.H. and Gadd, G.M. (1990) Metal Tolerance in Eukaryotic and Prokaryotic Algae In: Heavy Metal Tolerance in Plants: Evolutionary Aspects (Shaw, A.J., Ed.), pp. 105–118. CRC Press, Boca Raton, FL (Chapter 8).
[11] Kaplan, D., Heimer, Y.M., Abelovich, A. and Goldsbrough, P.B. (1995) Cadmium toxicity and resistance in *Chlorella sp.* Plant Sci. 109, 129–137.
[12] Zenk, M.H. (1996) Heavy detoxification in higher plants – a review. Gene 179, 21–30.
[13] Macfie, S.M. and Welburn, P.M. (2000) The cell wall as barrier to uptake of metals ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Arch. Environ. Contam. Toxicol. 39, 413–419.
[14] (a) Cobbett, C.S. (2000) Phytochelatins and their roles in heavy metal detoxification. Plant Physiol. 123, 825–832(b)Hall, J.L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.
[15] Mutoh, H. and Hayashi, Y. (1988) Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystins, small cadmium-binding peptides. Biochem. Biophys. Res. Commun. 151, 32–39.
[16] Mehra, R.K., Tarbet, E.B., Gray, W.R. and Winge, D.R. (1988) Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*. Proc. Natl. Acad. Sci. USA 85, 8815–8819.
[17] Mehra, R.K., Mulchandani, P. and Hunter, T.C. (1994) Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. Biochem. Biophys. Res. Commun. 200, 1193–1200.
[18] Gekeler, W., Grill, E., Winnacker, E.L. and Zenk, M.H. (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch. Microbiol. 150, 197–202.
[19] Mendoza-Cózatl, D., Devars, S., Loza-Tavera, H. and Moreno-Sánchez, R. (2002) Cadmium accumulation in the chloroplast of *Euglena gracilis*. Physiol. Plant. 115, 276–283.
[20] Grill, E., Winnacker, E.L. and Zenk, M.H. (1985) Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science 230, 674–676.
[21] (a)Clemens, S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475–486(b)Rea, P.A., Li, Z.S., Lu, Y.P. and Drozdowicz, Y.M. (1998) From vacuolar GS-X pumps to multispecific ABC transporters. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 727–760.
[22] Ortiz, D.F., Ruscitti, R., McCue, K.F. and Ow, D.W. (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J. Biol. Chem. 270, 4721–4728.
[23] Salt, D.E. and Rauser, W.E. (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol. 107, 1293–1301.
[24] Grill, E., Winnacker, E.L. and Zenk, M.H. (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants are functionally analogous to metallothioneins. Proc. Natl. Acad. Sci. USA 84, 439–443.

[25] Thumann, J., Grill, E., Winnacker, E.L. and Zenk, M.H. (1991) Reactivation of metal-requiring apoenzymes by phytochelatin–metal complexes. FEBS Lett. 284, 66–69.
[26] Meister, A. (1995) Glutathione metabolism. Methods Enzymol. 251, 3–13.
[27] Noctor, G. and Foyer, C. (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.
[28] Xiang, C. and Oliver, D. (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10, 1539–1550.
[29] Domínguez-Solís, J.R., Gutiérrez-Alcalá, G., Romero, L.C. and Gotor, C. (2001) The cytosolic *O*-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. J. Biol. Chem. 276, 9297–9302.
[30] Koprivova, A., Suter, M., den Camp, R.O., Brunold, C. and Kopriva, S. (2000) Regulation of sulfate assimilation by nitrogen in *Arabidopsis*. Plant Physiol. 122, 737–746.
[31] Harada, E., Yamaguchi, Y., Koizumi, N. and Sano, H. (2002) Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in *Arabidopsis*. J. Plant Physiol. 159, 445–448.
[32] Thomas, D. and Surdin-Kerjan, Y. (1997) Metabolism of sulphur amino acids in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 61, 503–532.
[33] Ono, B., Hazu, T., Yoshida, S., Kawato, T., Shinoda, S., Brzywczy, J. and Paszewski, A. (1999) Cysteine biosynthesis in *Saccharomyces cerevisiae*: a new outlook on pathway and regulation. Yeast 15, 1365–1375.
[34] Brzywczy, J., Sienko, M., Kucharska, A. and Paszewski, A. (2002) Sulphur amino acid synthesis in *Schizosaccharomyces pombe* represents a specific variant of sulphur metabolism in fungi. Yeast 19, 29–35.
[35] Marzluf, G.A. (1997) Molecular genetics of sulfur assimilation in filamentous fungi and yeast. Annu. Rev. Microbiol. 51, 73–96.
[36] Leustek, T. and Saito, K. (1999) Sulfate transport and assimilation in plants. Plant Physiol. 120, 637–643.
[37] Leustek, T., Martin, M.N., Bick, J.A. and Davies, J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 141–165.
[38] Grossman, A. and Takahashi, H. (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 163–210.
[39] Lass, B. and Ulrich-Eberius, C.I. (1984) Evidence for proton/sulfate cotransport and its kinetics in *Lemna gibba* G1. Planta 161, 53–60.
[40] Breton, A. and Surdin-Kerjan, Y. (1977) Sulfate uptake in *Saccharomyces cerevisiae*: biochemical and genetic study. J. Bacteriol. 132, 224–232.
[41] Cherest, H., Davidian, H.C., Thomas, D., Benes, V., Ansorge, W. and Surdin-Kerjan, Y. (1997) Molecular characterization of two high affinity sulfate transporters in *Saccharomyces cerevisiae*. Genetics 145, 627–635.
[42] Bussey, H., Storms, R.K., Ahmed, A., Albermann, K., Allen, E., Ansorge, W., Araujo, R., Aparicio, A., Barrell, B., Badcock, K., Benes, V., Botstein, D., Bowman, S., Bruckner, M., Carpenter, J., Cherry, J.M., Chung, E., Churcher, C., Coster, F., Davis, K., Davis, R.W., Dietrich, F.S., Delius, H., DiPaolo, T. and Hani, J., et al. (1997) The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XVI. Nature 387, 103–105
[43] Takahashi, H., Watanabe-Takahashi, A., Winith, F.W., Blake-Klaff, M., Hawkesford, M.J. and Saito, K. (2000) The roles of three different functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. Plant J. 23, 171–182
[44] Yoshimoto, N., Takahashi, H., Smith, F., Yamaya, T. and Saito, K. (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. Plant J. 29, 465–473.
[45] Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T. and Takahashi, H. (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in *Arobidopsis*. Plant Physiol. 131, 1511–1517.
[46] Takahashi, H., Yamazaki, M., Sasakura, N., Watanabe, A., Leustek, T., Engler, J. de A., Engler, G., Van Mongagu, M. and Saito, K. (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis tholiana*. Proc. Natl. Acad. Sci. USA 94, 11102–11107.
[47] Vidmar, J.J., Schjoerring, J.K., Touraine, B. and Glass, A.D.M. (1999) Regulation of the *hvst1* gene encoding a high-affinity sulfate transporter from *Hordeum vulgare*. Plant Mol. Biol. 40, 883–892.
[48] Vidmar, J.J., Tagmount, A., Cathala, N., Touraine, B. and Davidian, J.C. (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. FEBS Lett. 475, 65–69.
[49] (a)Yildiz, F.H., Davies, J.P. and Grossman, A.R. (1994) Characterization of sulfate transport in *Chlamydomonas reinhardtii* during sulfur-limited and sulfur-sufficient growth. Plant Physiol. 104, 981–987(b)Yildiz, F.H., Davies, J.P. and Grossman, A.R. (1996) Sulfur availabilty and the SAC1 gene control adenosine triphosphate sulfurylase gene expression in *Chlamydamonas reinhardtii*. Plant Physiol. 112, 669–675.
[50] Sahida, T., Stern, A., Lee, D. and Schiff, J.A. (1985) Localization of a sulphate-activating system within *Euglena* mitochondria. Biochem. J. 232, 357–365.
[51] Chen, H.-Ch., Yokthongwattana, K., Newton, A.J. and Melis, A. (2003) *SulP*, a nuclear gene encoding a putative chloroplast-targeted sulfate permease in *Chlamydomonas reinhardtii*. Planta 218, 98–106.
[52] Ohta, N., Matsuzaki, M., Misumi, O., Miyagishima, S., Nozaki, H., Tanaka, K., Shin-i, T., Kohara, Y. and Kuroiwa, T. (2003) Complete sequence and analysis of the plastid genome of the unicellular red alga *Cyanidioschyzon merolae*. DNA Res. 10, 67–77.
[53] Takahashi, H., Asanuma, W. and Saito, K. (1999) Cloning of an *Arabidopsis* cDNA encoding a chloroplast localizing sulphate transporter isoform. J. Exp. Bot. 50, 1713–1714.
[54] Lee, S. and Leustek, T. (1998) APS kinase from *Arabidopsis thaliana*: genomic organization, expression, and kinetic analysis of the recombinant enzyme. Biochem. Biophys. Res. Commun. 247, 171–175.
[55] Hell, R. (1997) Molecular physiology of plant sulfur metabolism. Planta 202, 138–148.
[56] Osslund, T., Chandler, C. and Segel, I. (1982) ATP sulfurylase from higher plants. Plant Physiol. 70, 39–45.
[57] Cornish-Bowden, A. and Cardenas, M.L. (2001) Information transfer in metabolic pathways. Eur. J. Biochem. 268, 6616–6624.
[58] Ullrich, T.C., Blaesse, M. and Huber, R. (2001) Crystal structure of ATP sulfurylase from *Saccharomyces cerevisiae*, a key enzyme in sulfate activation. EMBO J. 20, 316–329.
[59] MacRae, I.J., Segel, I.H. and Fisher, A.J. (2001) Crystal structure of ATP sulfurylase from *Penicillium chrysogenum*: insights into the allosteric regulation of sulfate assimilation. Biochemistry 40, 6795–6804.
[60] Renosto, F., Martin, R.L., Wailes, L.M., Daley, L.A. and Segel, I.H. (1990) Regulation of inorganic sulfate activation in filamentous fungi. Allosteric inhibition of ATP sulfurylase by 3′-phosphoadenosine-5′-phosphosulfate. J. Biol. Chem. 265, 10300–10308.

[61] Renosto, F., Patel, H.C., Martin, R.L., Thomassian, C., Zimmerman, G. and Segel, I.H. (1993) ATP sulfurylase from higher plants: kinetic and structural characterization of the chloroplast and cytosol enzymes from spinach leaf. Arch. Biochem. Biophys. 307, 272–285.

[62] Rotte, C. and Leustek, T. (2000) Differential subcellular localization and expression of ATP sulfurylase and 5′-adenylylsulfate reductase during ontogenesis of *Arabidopsis* leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions. Plant Physiol. 124, 715–724.

[63] Li, J., Saidha, T. and Schiff, J.A. (1991) Purification and properties of two forms of ATP sulfurylase from *Euglena*. Biochim. Biophys. Acta 1078, 68–76.

[64] Logan, H.M., Cathala, N., Grignon, C. and Davidian, J.C. (1996) Cloning of a cDNA encoded by a member of the *Arabidopsis thaliana* ATP sulfurylase multigene family. J. Biol. Chem. 271, 12227–12233.

[65] Klonus, D., Hofgen, R., Willmitzer, L. and Riesmeier, J.W. (1994) Isolation and characterization of two cDNA clones encoding ATP-sulfurylases from potato by complementation of a yeast mutant. Plant J. 6, 105–112.

[66] Hatzfeld, Y., Cathala, N., Grignon, C. and Davidian, J.C. (1998) Effect of ATP sulfurylase overexpression in Bright yellow 2 tobacco cells. Plant Physiol 116, 1307–1313.

[67] Murillo, M. and Leustek, T. (1995) Adenosine-5′-triphosphate-sulfurylase from *Arabidopsis thaliana* and *Escherichia coli* are functionally equivalent but structurally and kinetically divergent: nucleotide sequence of two adenosine-5′-triphosphate-sulfurylase cDNAs from *Arabidopsis thaliana* and analysis of a recombinant enzyme. Arch. Biochem. Biophys. 323, 195–204.

[68] Nozaki, T., Arase, T., Shigeta, Y., Asai, Y., Leustek, T. and Takeuchi, T. (1998) Cloning and bacterial expression of adenosine-5′-triphosphate sulfurylase from the enteric protozoan parasite *Entamoeba histolytica*. Biochim. Biophys. Acta 1429, 284–291.

[69] Bick, J.A., Aslund, F., Chen, Y. and Leustek, T. (1998) Glutaredoxin function for the carboxyl-terminal domain of the plant-type 5′-adenylylsulfate reductase. Proc. Natl. Acad. Sci. USA 95, 8404–8409.

[70] Gutierrez-Marcos, J.F., Roberts, M.A., Campbell, E.I. and Wray, J.L. (1996) Three members of a novel small gene-family from *Arabidopsis thaliana* able to complement functionally an *Escherichia coli* mutant defective in PAPS-reductase activity encode proteins with a thioredoxin-like domain and APS reductase activity. Proc. Natl. Acad. Sci. USA 93, 13377–13382.

[71] Lillig, C.H., Schiffmann S, Berndt, C. and Berken, A. (2001) Molecular and catalytic properties of *Arabidopsis thaliana* adenylyl sulfate (APS)-kinase. Arch. Biochem. Biophys. 392, 303–310.

[72] Korch, C., Mountain, H.A. and Bystrom, A.S. (1991) Cloning, nucleotide sequence, and regulation of *MET14*, the gene encoding the APS kinase of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 229, 96–108.

[73] Thomas, D., Barbey, R. and Surdin-Kerjan, Y. (1990) Gene-enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae*. Study of the 3′-phosphoadenylylsulfate reductase structural gene. J. Biol. Chem. 265, 15518–15524.

[74] Kopriva, S., Buchert, T., Fritz, G., Suter, M., Benda, R., Schunemann, V., Koprivova, A., Schurmann, P., Trautwein, A.X., Kroneck, P.M. and Brunold, C. (2002) The presence of an iron–sulfur cluster in adenosine 5′-phosphosulfate reductase separates organisms utilizing adenosine 5′-phosphosulfate and phosphoadenosine 5′-phosphosulfate for sulfate assimilation. J. Biol. Chem. 277, 21786–21791.

[75] Foster, B.A., Thomas, S.M., Mahr, J.A., Renosto, F., Patel, H.C. and Segel, I.H. (1994) Cloning and sequencing of ATP sulfurylase from *Penicillium chrysogenum*. Identification of a likely allosteric domain. J. Biol. Chem. 269, 19777–19786.

[76] Koprivova, A., Meyer, A.J., Schween, G., Herschbach, C., Reski, R. and Kopriva, S. (2002) Functional knockout of the adenosine 5-phosphosulfate reductase gene in *Physcomitrella patens* revives an old route of sulfate assimilation. J. Biol. Chem. 277, 32195–32201.

[77] Gao, Y., Schofield, O.M.E. and Leustek, T. (2000) Characterization of sulfate assimilation in marine algae focusing on the enzyme 5′-adenylylsulfate reductase. Plant Physiol. 123, 1087–1096.

[78] Saidha, T., Na, S.Q., Li, J. and Schiff, J.A. (1988) A sulphate metabolizing centre in *Euglena* mitochondria. Biochem. J. 253, 533–539.

[79] Nakayama, M., Akashi, T. and Hase, T. (2000) Plant sulfite reductase: molecular structure, catalytic function and interaction with ferredoxin. J. Inorg. Biochem. 82, 27–32.

[80] Kobayashi, K. and Yoshimoto, A. (1982) Studies on yeast sulfite reductase IV. Structure and steady state kinetics. Biochim. Biophys. Acta 705, 348–356.

[81] Yonekura-Sakakibara, K., Ashikari, T., Tanaka, Y., Kusumi, T. and Hase, T. (1998) Molecular characterization of tobacco sulfite reductase: enzyme purification, gene cloning, and gene expression analysis. J. Biochem. 124, 615–621.

[82] Bork, C., Schwenn, J.D. and Hell, R. (1998) Isolation and characterization of a gene for assimilatory sulfite reductase from *Arabidopsis thaliana*. Gene 212, 147–153.

[83] Brühl, A., Haverkamp, T., Gisselmann, G. and Schwenn, J.D. (1996) A cDNA clone from *Arabidopsis thaliana* encoding plastidic ferredoxin:sulfite reductase. Biochim. Biophys. Acta. 1295, 119–124.

[84] Hell, R., Jost, R., Berkowitz, O. and Wirtz, M. (2002) Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. Amino Acids 22, 245–257.

[85] Cherest, H. and Surdin-Kerjan, Y. (1992) Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. Genetics 130, 51–58.

[86] Soda, K. (1987) Microbial sulfur amino acids: an overview. Methods Enzymol. 143, 453–459.

[87] Saito, K. (2000) Regulation of sulfate transport and synthesis of sulfur containing amino acids. Curr. Opin. Plant Biol. 3, 188–195.

[88] Höfgen, R., Dreft, O., Willmitzer, L. and Hesse, H. (2001) Manipulation of thiol contents in plants. Amino Acids 20, 291–299.

[89] Nozaki, T., Shigeta, Y., Saito-Nakano, Y., Imada, M. and Kruger, W.D. (2001) Characterization of transsulfuration and cysteine biosynthetic pathways in the protozoan hemoflagellate, *Trypanosoma cruzi*. Isolation and molecular characterization of cystathionine β-synthase and serine acetyltransferase from *Trypanosoma*. J. Biol. Chem. 276, 6516–6523.

[90] Hesse, H., Lipke, J., Altmann, T. and Hofgen, R. (1999) Molecular cloning and expression analyses of mitochondrial and plastidic isoforms of cysteine syuthase (*O*-acetylserine(thiol)lyase) from *Arabidopsis thaliana*. Amino Acids 16, 113–131.

[91] Jost, R., Berkowitz, O., Wirtz, M., Hopkins, L., Hawkesford, M.J. and Hell, R. (2000) Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene 253, 237–247.

[92] Noctor, G., Arisi, A.C., Jouanin, L. and Foyer, C. (1998) Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol. 118, 471–482.

[93] Foyer, C.H., Theodoulou, F.L. and Delrot, S. (2001) The functions of inter- and intracellular glutathione transport systems in plants. Trends Plant Sci. 6, 486–492.
[94] Cooper, A.J.L. (1983) Biochemistry of sulfur-containing amino acids. Annu. Rev. Biochem. 52, 187–222.
[95] May, J.M. and Leaver, C.J. (1994) *Arabidopsis thaliana* γ-glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *Escherichia coli* homologs. Proc. Natl. Acad. Sci. USA 91, 10059–10063.
[96] Soltaninassab, S.R., Sekhar, K.R., Meredith, M.J. and Freeman, M.L. (2000) Multi-faceted regulation of γ-glutamylcysteine synthetase. J. Cell. Physiol. 182, 163–170.
[97] Lueder, D.V. and Phillips, M.A. (1996) Characterization of *Trypanosoma brucei* γ-glutamylcysteine synthetase, an essential enzyme in the biosynthesis of thrypanothione (diglutathionylspermidine). J. Biol. Chem. 271, 17485–17490.
[98] Griffith, O.W. (1982) Mechanisms of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem. 257, 13704–13712.
[99] Huang, C.S., Moore, W.R. and Meister, A. (1988) On the active site thiol of γ-glutamylcysteine synthetase: relationships to catalysis, inhibition, and regulation. Proc. Natl. Acad. Sci. USA 85, 2464–2468.
[100] Kelly, B.S., Antholine, W.E. and Griffith, O.W. (2001) *Escherichia coli* γ-glutamylcysteine synthetase. J. Biol. Chem. 277, 50–58.
[101] Griffith, O.W. and Mulcahy, R.T. (1999) The enzymes of glutathione synthesis: γ-glutamylcysteine synthetase. Adv. Enzymol. Relat. Areas Mol. Biol. 73, 209–267.
[102] Wang, C.L. and Oliver, D.J. (1997) Glutathione synthetase: similarities of the proteins from *Schizosaccharomyces pombe* and *Arabidopsis thaliana*. Biochem. J. 326, 563–566.
[103] Rawlins, M.R., Leaver, J.C. and May, M.J. (1995) Characterization of an *Arabidopsis thaliana* cDNA encoding glutathione synthetase. FEBS Lett. 376, 81–86.
[104] Meierjohann, S., Walter, R.D. and Muller, S. (2002) Glutathione synthetase from *Plasmodium falciparum*. Biochem. J. 363, 833–838.
[105] Kneer, R., Kutchan, T.M., Hochberger, A. and Zenk, M.H. (1992) *Saccharomyces cerevisiae* and *Neurospora crassa* contain heavy metal sequestering phytochelatin. Arch. Microbiol. 157, 305–310.
[106] Oven, M., Page, J.E., Zenk, M.H. and Kutchan, T.M. (2002) Molecular characterization of the homophytochelatin synthase of soybean *Gycine max*. J. Biol. Chem. 277, 4747–4754.
[107] Kubota, H., sato, K., Yamada, T. and Maitani, T. (2000) Phytochelatin homologs induced in hairy roots of horseradish. Phytochemistry 53, 239–245.
[108] Avilés, C., Loza-Tavera, H., Terry, N. and Moreno-Sánchez, R. (2003) Mercury pretreatment selects an enhanced cadmium accumulating phenotype in *Euglena gracilis*. Arch. Microbiol. 180, 1–10.
[109] Berlich, M., Menge, S., Bruns, I., Schimdt, J., Schneider, B. and Krauss, G.J. (2002) Coumarins give misleading absorbance with Ellman's reagent suggestive of thiol conjugates. Analyst 127, 333–336.
[110] Bruns, I., Sutter, K., Menge, S., Neumann, D. and Krauss, G.J. (2001) Cadmium let increase the glutathione pool in bryophytes. J. Plant Physiol. 158, 79–89.
[111] Vatamaniuk, O.K., Mari, S., Lu, Y. and Rea, P.A. (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. J. Biol. Chem. 275, 31451–31459.
[112] Vatamaniuk, O.K., Mari, S., Lang, A., Chalasani, S., Demkiv, L.O. and Rea, P.A. (2004) Phytochelatin synthase, a dipeptidyltransferase that undergoes multisite acylation with γ-glutamylcysteine during catalysis. J. Biol. Chem. 279, 22449–22460.
[113] Grill, E., Löffler, S., Winnacker, E.L. and Zenk, M.H. (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc. Natl. Acad. Sci. USA 86, 6838–6842.
[114] Ha, S., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B. and Cobbett, C.S. (1999) Phytochelatin synthase genes from *Arabidopsis* and yeast *Schizosaccharomyces pombe*. Plant Cell 11, 1153–1163.
[115] Rauser, W.E. (1990) Phytochelatins. Annu. Rev. Biochem. 59, 61–86.
[116] Clemens, S., Kim, E.J., Neumann, D. and Schroeder, J. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J. 18, 3325–3333.
[117] Heiss, S., Wachter, A., Bogs, J., Cobbett, C. and Rausch, T. (2003) Phytochelatin synthase (PCS) protein is induced in *Brassica juncea* leaves after prolonged Cd exposure. J. Exp. Bot. 54, 1833–1839.
[118] Vatamaniuk, O.K., Bucher, E.A., Ward, J,T. and Rea, P.A. (2001) A new pathway for heavy metal detoxification in animals. Phytochelatin synthase is required for cadmium tolerance in *Caenorhabditis elegans*. J. Biol. Chem. 276, 20817–20820.
[119] Clemens, S., Schroeder, J.I. and Degenkolb, T. (2001) *Caenorhabditis elegans* expresses a functional phytochelatin synthase. Eur. J. Biochem. 268, 3640–3643.
[120] Cobbett, C.S. (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr. Opin. Plant Biol. 3, 211–216.
[121] Ruotolo, R., Peracchi, A., Bolchi, A., Infusini, G., Amoresano, A. and Ottonello, S. (2004) Domain organization of phytochelatin synthase. J. Biol. Chem. 279, 14686–14693.
[122] Howden, R., Goldsbrough, P.B., Andersen, C.R. and Cobbett, C.S. (1995) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. 107, 1059–1066.
[123] Maier, T., Yu, C., Kullertz, G. and Clemens, S. (2003) Localization and functional characterization of metal-binding sites in phytochelatin synthases. Planta 218, 300–308.
[124] Hayashi, Y., Nakagawa, C.W., Mutoh, N., Isobe, M. and Goto, T. (1991) Two pathways in the biosynthesis of cadystins (gamma EC)nG in the cell-free system of the fission yeast. Biochem. Cell Biol. 69, 115–121.
[125] Al-Lahham, A., Rohde, V., Heim, P., Leuchter, R., Veeck, J., Wunderlich, C., Wolf, K. and Zimmermann, M. (1999) Biosynthesis of phytochelatins in the fission yeast. Phytochelatin synthesis: a second role for the glutathione synthetase gene of *Schizosaccharomyces pombe*. Yeast 15, 385–396.
[126] Imai, K., Obata, H., Shimizu, K. and Komiya, T. (1996) Conversion of glutathione into cadystins and their analogs catalyzed by carboxypeptidase Y. Biosci. Biotechnol. Biochem. 60, 1193–1194.
[127] Ono, B., Kijima, K., Ishii, N., Takahiro, K., Matsuda, A., Paszewski, A. and Shinoda, S. (1996) Regulation of sulphate assimilation in *Saccharomyces cerevisiae*. Yeast 12, 1153–1162.
[128] Takagi, H., Yoshioka, K., Awano, N., Nakamori, S. and Ono, B. (2003) Role of *Saccharomyces cerevisiae* serine *O*-acetyltransferase in cysteine biosynthesis. FEMS Microbiol. Lett. 218, 291–297.
[129] Noji, M. and Saito, K. (2002) Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolic engineering in plants. Amino Acids 22, 231–243.
[130] Richman, P.G. and Meister, A. (1975) Regulation of γ-glutamylcysteine synthetase by non-allosteric feedback inhibition by glutathione. J. Biol. Chem. 250, 1422–1426.
[131] Arisi, A.C., Noctor, G., Foyer, C.H. and Jouanin, L. (1997) Modification of thiol contents in poplars (*Populus tremula* × *P. alba*) overexpressing enzymes involved in glutathione synthesis. Planta 203, 272–362

[132] Creissen, G., Firmin, J., Fryer, M., Kular, B., Leyland, N., Reynolds, H., Pastori, G., Wellburn, F., Baker, N., Wellburn, A. and Mullineaux, P. (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell 11, 1277–1291.

[133] Hell, R. and Bergmann, L. (1990) γ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta 180, 603–612.

[134] Noctor, G., Arisi, A.C.M., Jouanin, L., Valadier, M.H., Roux, Y. and Foyer, H. (1997) The role of glycine in determining the rate of glutathione synthesis in poplar. Posible implications for glutathione production during stress. Physiol. Plant 100, 255–263.

[135] Rauser, W.E. and Meuwly, P. (1995) Retention of cadmium in roots of maize seedlings. Plant Physiol. 109, 195–202.

[136] Momose, Y. and Iwahashi, H. (2003) Bioassay of cadmium using a DNA microarray: genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. Environ. Toxicol. Chem. 20, 2353–2360.

[137] Fauchon, M., Lagniel, G., Aude, J.C., Lombardia, L., Soularue, P., Petat, C., Marguerie, G., Sentenac, A., Werner, M. and Labarre, J. (2002) Sulfur sparing in the yeast proteome in response to sulfur demand. Mol. Cell 9, 713–723.

[138] Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M. and Saito, K. (2003) Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of *O*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J. 33, 651–663.

[139] Smith, F.W., Hawkesford, M.J., Ealing, P.M., Clarkson, D.T., Vanden Berg, P.J., Belcher, A., R and Warrilow, A.G. (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J. 12, 875–884.

[140] Heiss, S., Schäfer, H.J., Haag-Kerwer, A. and Rausch, T. (1999) Cloning sulfur assimilation genes of *Brassica juncea* L.: cadmium differentially affects the expression of a putative low-affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. Plant Mol. Biol. 39, 847–857.

[141] Nocito, F.F., Pirovano, L., Cocucci, M. and Sacchi, G.A. (2002) Cadmium-induced sulfate uptake in maize roots. Plant Physiol. 129, 1872–1879.

[142] Vido, K., Spector, D., Lagniel, G., Lopez, S., Toledano, M. and Labarre, J. (2001) A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. J. Biol. Chem. 276, 8469–8474.

[143] Stephen, D.W. and Jamieson, D.J. (1997) Amino acid-dependent regulation of the *Saccharomyces cerevisiae* GSH1 gene by hydrogen peroxide. Mol. Microbiol. 23, 203–210.

[144] Dormer, U.H. Westwater, J., Stephen, D.W., S and Jamieson, D.J. (2002) Oxidant regulation of the *Saccharomyces cerevisiae GSH1* gene. Biochim. Biophys. Acta 1576, 23–29.

[145] Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kiviven, K., Brazma, A., Jones, N. and Bähler, J. (2003) Global transcriptional responses of fission yeast to enviromental stress. Mol. Cell Biol. 14, 214–229.

[146] Schäfer, H., Haag-Kerwer, A. and Rausch, T. (1998) cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L.: evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. Plant Mol. Biol. 37, 87–97.

[147] May, M.J., Vernoux, T., Sánchez-Fernandez, R., Montagu, M.V. and Ine, D. (1998) Evidence for posttranscriptional activation of γ-glutamylcysteine synthetase during plant stress responses. Proc. Natl. Acad Sci. USA 95, 12049–12054

[148] Lee, S. and Korban, S. (2002) Transcriptional regulation of *Arabidopsis thaliana* phytochelatin synthase (AtPCS1) by cadmium during early stages of plant development. Planta 215, 689–693.

[149] Hansen, J. and Johannesen, P.F. (2000) Cysteine is essential for transcriptional regulation of the sulfur assimilation genes in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 263, 535–542.

[150] Bolchi, A., Petrucco, S., Tenca, P.L., Foroni, C. and Ottonello, S. (1999) Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. Plant Mol. Biol. 39, 527–537.

[151] Lappartient, A.G., Vidmar, J.J., Leustek, T., Glass, A.D. and Touraine, B. (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J. 18, 89–95.

[152] Vauclare, P., Kopriva, S., Fell, D., Suter, M., Sticher, L., von Ballmoos, P., Krahenbuhl, U., den Camp, R.O. and Brunold, C. (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J. 31, 729–740.

[153] Blaiseau, P.L. and Thomas, D. (1998) Multiple transcriptional activation complexes tether the yeast activator Met4 to DNA. EMBO J. 17, 6327–6336.

[154] Dormer, U.H., Westwater, J., McLaren, N.F., Kent, N.A., Mellor, J. and Jamieson, D.J. (2000) Cadmium-inducible expression of the yeast GSH1 gene requires a functional sulfur-amino acid regulatory network. J. Biol. Chem. 275, 32611–32616.

[155] Kuras, L., Barbey, R. and Thomas, D. (1997) Assembly of a bZIP-bHLH transcription activation complex: formation of the yeast Cbf1–Met4–Met28 complex is regulated through Met28 stimulation of Cbf1 DNA binding. EMBO J. 16, 2441–2451.

[156] Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J. and Toledano, M.B. (1997) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J. Biol. Chem. 274, 16040–16046.

[157] Wu, A.L. and Moye-Rowley, W.S. (1994) GSH1, which encodes gamma-glutamylcysteine, is a target gene for Yap1 transcriptional regulation. Mol. Cell Biol. 14, 5832–5839.

[158] Li, Z.-S., Lu, Y.-P., Zhen, R.-G., Szczypka, M., Thiele, D.J and Rea, P.A. (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato) cadmium. Proc. Natl. Acad. Sci. USA 94, 42–47.

[159] Salt, D.E. and Wagner, G.J. (1993) Cadmium transport across tonoplast of vesicles from oat roots. J. Biol. Chem. 17, 12297–12302.

[160] Speiser, D.M., Ortiz, D.F., Kreppel, L., Scheel, G., McDonald, G. and Ow, D.W. (1992) Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. Mol. Cell Biol. 12, 5301–5310.

[161] Juang, R.H., McCue, K.F. and Ow, D.W. (1993) Two purine biosynthetic enzymes required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate in vitro. Arch. Biochem. Biophys. 304, 392–401.

[162] Wu, J.S., Sung, H.Y. and Juang, R.H. (1995) Transformation of cadmium-binding complexes during cadmium sequestration in fission yeast. Biochem. Mol. Biol. Inter. 36, 1169–1175.

[163] Vande, J.G. and Ow, D.W. (2001) Accumulation of metal-binding peptides in fission yeast requires *hmt2*$^{+}$. Mol. Microbiol. 42, 29–36.

[164] Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G. and Ow, D.W. (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J. 11, 3491–3499.

[165] Li, Z.S., Szczypka, M., Lu, Y.P., Thiele, D.J. and Rea, P.A. (1996) The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. J. Biol. Chem. 271, 6509–6517.

[166] Sharma, K.G., Mason, D.L., Liu, G., Rea, P.A., Bachhawat, A.K. and Michaelis, S. (2002) Localization, regulation, and substrate transport properties of Bpt1p, a *Saccharomyces cerevisiae* MRP-type ABC transporter. Eukaryot. Cell. 1, 391–400.

[167] Sánchez-Fernández, R., Davies, T.G., Coleman, J.O. and Rea, P.A. (2001) The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J. Biol. Chem. 276, 30231–30244.

[168] MacDiarmid, C.W., Milanick, M.A. and Eide, D.J. (2002) Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J. Biol. Chem. 277, 39187–39194.

[169] Williams, L.E., Pittman, J.K. and Hall, J.L. (2000) Emerging mechanisms for heavy metal transport in plants. Biochim. Biophys. Acta 1465, 104–126.

[170] Guerinot, M.L. (2000) The ZIP family of metal transporters. Biochim. Biophys. Acta 1465, 190–198.

[171] Mäser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, J.M., Sanders, D., Harper, J., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D., Kim, S.A. and Guerinot, M.L. (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiol. 126, 1646–1667.

[172] Liu, X.F., Supek, F., Nelson, N. and Culotta, V.C. (1997) Negative control of heavy metal uptake by the *Saccharomyces cerevisiae* BSD2 gene. J. Biol. Chem. 272, 11763–11769.

[173] Liu, X.F. and Culotta, V.C. (1999) Post-translation control of Nramp metal transport in yeast. Role of metal ions and the BSD2 gene. J. Biol. Chem. 274, 4863–4868.

[174] Conklin, D.S., McMaster, J.A., Culbertson, M.R. and Kung, C. (1992) COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol. Cell Biol. 12, 3678–3688.

[175] Conklin, D.S., Culbertson, M.R. and Kung, C. (1994) Interactions between gene products involved in divalent cation transport in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 244, 303–311.

[176] MacDiarmid, C.W., Gaither, L.A. and Eide, D. (2000) Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. EMBO J. 19, 2845–2855.

[177] MacDiarmid, C.W., Milanick, M.A. and Eide, D.J. (2003) Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J. Biol. Chem. 279, 15065–15072.

[178] Bloß, T., Clemens, S. and Nies, D.H. (2002) Characterization of the ZAT1p zinc trasnporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes. Planta 214, 783–791.

[179] Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L. and Wagner, G.J. (2000) Expression of *Arabidopsis CAX2* in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol. 124, 125–133.

[180] Meuwly, P. and Rauser, W.E. (1992) Alteration thiol pools in roots and shoots in maize seedlings exposed to cadmium. Plant Physiol. 99, 8–15.

[181] Barbas, J., Santhanagopalan, V., Blaszczynski, W., Elli, W.R. and Winge, D.R. (1992) Conversion in the peptides coating cadmium:sulfide crystallites in *Candida glabrata*. J. Inorg. Biochem. 48, 95–105.

[182] Dameron, C.T., Smith, B.R. and Winge, D.R. (1989) Glutathione-coated cadmium-sulfide crystallites in *Candida glabrata*. J. Biol. Chem. 264, 17355–17360.

[183] Coppelloti, O. (1989) Glutathione, cysteine and acid-soluble thiol levels in *Euglena gracilis* cells exposed to copper and cadmium. Comp Biochem. Physiol. 94C, 35–40.

[184] Nagel, K., Adelmeier, U. and Voigt, J. (1996) Subcellular distribution of cadmium in the unicellular green alga *Chamydomonas reinhardtii*. J. Plant Physiol. 149, 86–90.

[185] Carr, H.P., Cario, A., Yang, M.S. and Wong, M.H. (1998) Characterization of the cadmium-binding capacity of *Chlorella vulgaris*. Bull. Environ. Contam. Toxicol. 60, 433–440.

[186] Tsuji, N., Hirayanagi, N., Okada, M., Miyasaka, H., Hirata, K., Zenk, M.H. and Miyamoto, K. (2002) Enhancement of tolerance to heavy metals and oxidative stress in *Dunaliella tertiolecta* by Zn-induced phytochelatin synthesis. Biochem. Biophys. Res. Commun. 293, 653–659.

[187] Mehta, S.K. and Gaur, J.P. (1999) Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. New Phytol. 143, 253–259.

[188] Sripornadulsil, S., Traina, S., Verma, D.P.S. and Sayre, R.T. (2002) Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 14, 2837–2847.

[189] Alia, Mohanty, P. and Matysik, J. (2001) Effect of proline on the production of singlet oxygen. Amino Acids 21, 195–200.

[190] Zhu, Y.L., Pilon-Smits, E.A.H., Tarun, A.S., Weber, S.U., Jouanin, L. and Terry, N. (1999) Cadmium tolerance and accumulation in indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol. 121, 1169–1177.

[191] Zhu, Y.L., Pilon-Smits, E.A.H., Tarun, A.S., Jouanin, L. and Terry, N. (1999) Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. Plant Physiol. 119, 73–79.

[192] Grant, C.M., MacIver, F.H. and Dawes, I.W. (1997) Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide γ-glutamylcysteine. Mol. Biol. Cell 8, 1699–1707.

[193] Kim, S.J., Shin, Y.H., Park, E.H., Sa, J.H. and Lim, C.J. (2003) Regulation of the gene encoding glutathione synthetase from fission yeast. J. Biochem. Mol. Biol. 36, 326–331.

[194] Lee, S., Moon, J.S., Ko, T.S., Petros, D., Goldsbrough, P.B. and Korban, S.S. (2003) Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol. 131, 656–663.

[195] Hawes, C.S. and Nicholas, D.J.D. (1973) Adenosine 5′-triphosphate sulphurylase from *Saccharomyces cerevisiae*. Biochem. J. 133, 541–550.

[196] Schriek, U. and Schwenn, J.D. (1986) Properties of the purified APS-kinase from *Escherichia coli* and *Saccharomyces cerevisiae*. Arch. Microbiol. 145, 32–38.

[197] Schwenn, J.D., Krone, F.A. and Husmann, K. (1988) Yeast PAPS reductase: properties and requirements of the purified enzyme. Arch. Microbiol. 150, 313–319.

[198] Ono, B., Kijima, K., Inoue, T., Miyoshi, S., Matsuda, A. and Shinoda, S. (1994) Purification and properties of *Saccharomyces cerevisiae* cystathionine β- synthase. Yeast 10, 333–339.

[199] Yamagata, S., D'Andrea, R.J., Fujisaki, S., Isaji, M. and Nakamura, K. (1993) Cloning and bacterial expression of the CYS3 gene encoding cystathionine γ-lyase of *Saccharomyces cerevisiae* and the physicochemical and enzymatic properties of the protein. J. Bacteriol. 175, 4800–4808.

[200] Dennda, G. and Kula, M.R. (1986) Purification and evaluation of the glutathione-synthesizing enzymes from *Candida boidinni* for cell-free synthesis of glutathione. J. Biotechnol. 4, 143–158.

De: <fems-reviews@bham.ac.uk>
Enviado el: Domingo, 06 de Junio de 2004 11:15:59 a.m.
Para: cozatl@hotmail.com, davidmendozac@netscape.net
CC: fems-reviews@bham.ac.uk, femsre@fems-microbiology.org
Asunto: FEMSRE-04-03-0011 / Major revision

06-Jun-2004

Dear Dr. David Mendoza-Cozatl,

Manuscript id: FEMSRE-04-03-0011
Title: Sulfur Assimilation and Glutathione Metabolism Under Cadmium Stress in Yeast, Photosynthetic Protists and Plants
Authors: Mendoza-Cozatl, David; Hernández, Andrea; Loza, Herminia; Moreno-Sánchez, Rafael
Submitted to: FEMS Microbiology Reviews

Thank you for submitting the above manuscript to our journal. It has now been expertly refereed, and the recommendations made are listed below. I apologise for the length of time this has taken.

Both reviewers were asked to look at both manuscripts. Reviewer 1 had recommended combining the manuscripts, not knowing the background to this resubmission; obviously, I am not going to ask you to do this. Reviewer 2 has been unable yet to provide comments on the FEMSRE-04-03-0012 and has promised to do so shortly. I hope to be able to contact you about the second manuscript soon.

My major comment is that this review is not particularly evaluative. I would like you to make the review a bit sharper are more critical in its content. We rely on our authors to evaluate and interpret data for the general reader and provide a dynamic and interesting review.

Both reviewers raise a number of concerns. I would like you to address the missing data as requested by Reviewer 1 and to address the points made by Reviewer 2. If possible, you should seek some help with the English. If that is not possible, it will be edited here, but that will slow down the process. I have not edited the manuscript at this stage.

I am willing to accept a major revision of the review addressing these points. This may then need to be reviewed again.

- Please return your revised manuscript to me within two months if at all possible, as this will expedite publication.

- With your revision please enclose an e-mail answering all the points made by the referees and myself in a numbered list. You can conveniently do this through the button "View and Respond to Comments" in your Submitting Author Center.

- You should send your revised manuscript via your Submitting Author Center at http://mc.manuscriptcentral.com/femsre. For this purpose you will find that you can click "create revision" in the list of manuscripts with a decision. Please note that this option will disappear 90 days from today.

- If your paper will be accepted, the electronic files will in principle be used for producing the final publication, but the publisher Elsevier may request a set of high-quality print-outs of your figures for production purposes.

I am looking forward to receiving your revision in due course, please let me know if you decide not to submit a revision or for some reason cannot respond within the two months suggested above.

Sincerely,

Prof. Nigel Brown
Editor
FEMS Microbiology Reviews

P.S. Please ensure that only one response is made to this e-mail and that this should come through the corresponding author and combine the views of all authors.

Reviewer's Comments to Authors:

Reviewer: 1
Comments to the Author: The paper don't meet all requirements necessary for a critical review of the present scientific knowledge in the field sulfate assimilation/glutathione/phytochelatins.

Recent papers showing induction and compartmentations in algae and fungi are not taken into consideration; e.g. results with Candida glabrata and Schizosaccharomyces pombe are missing.

There needs to be more data provided on the molecular biology of the systems.

p17: No phytochelatins were detected in Caenorhabiditis elegans.

Data on variants of phytochelatins are incomplete; Glutamine has also been reported as a terminal amino acid in plants.

The authors need to check on which organisms phytochelatins have been clearly identified. Coumarins give misleading absorbances with Ellman's reagent, suggestive of thiol conjugates. (Analyst 127, 333-336, 2002)

Reviewer: 2
Comments to the Author: This review is much improved now that it has largely been separated from the metabolic control analysis (MCA). With some help with the English from the editor and/or editorial assistant(s), this manuscript is now probably suitable for publication in FEMS Microbiology Reviews. It brings together a huge amount of research and provides several very informative diagrams that will be a useful resource for many people working in this and adjacent research areas.

The following points are raised to help the authors further clarify and improve the text:

p. 5, last line:
The implication here, which is probably not correct, is that plants have the facility to transport Cd.GS complexes into the vacuole directly, as is the case for the fission and budding yeast Schizosaccharomyces pombe and Saccharomyces cerevisiae, respectively. No evidence to this effect has been published (and, in fact, there are two sets of unpublished data from different groups that contradict this idea). This is, of course, not to say that plants do not have the facility for catalyzing the vacuolar uptake of Cd.PC complexes.

p. 13, line 6 from bottom:
"posses" should read "possesses".

p. 17, line 7 from bottom:
The authors might want to update this section by consideration of two new papers - Ruotolo et al (2004) J. Biol. Chem. 279: 14686-14693 and Vatamaniuk et al (2004) J. Biol. Chem. 22449-22460 dealing with the domain organization and catalytic mechanism of PCS, respectively.

p. 18, last para.:
Have PCs been unequivocally demonstrated in S. cerevisiae and Neurospora crassa? If they are there, they are present at levels below the detection limits of the techniques that most investigators use.

p. 20, line 10:
The authors conclude that the yeast Cd2+ response is essentially regulated at the transcriptional level. Is this in fact the case? That is, do the results from the transcriptomic and proteomic analyses agree in the case of each and every gene and its transcription and translation products? It would be a more accurate to point out that in many cases this is not so and in most cases it is simply not known.

p. 21, para. 3:
"HMT1 is specific for phytochelatins transport…" should read "HMT1 is specific for phytochelatin transport". The sentence after this one should also be reworded for clarity.

p. 22, para. 1:
Li et al (1997) not Ortiz et al (1995) established that YCF1 does not transport phytochelatins.

p. 25 - Protists and Unicellular Algae:
It might be wise for the authors to be more circumspect concerning the localization of phytochelatins to chloroplasts and/or mitochondria in these organisms. The data they discuss are equivocal. My impression is that there is a brake in style here in that the authors devote a lot more the discussion to Euglena gracilis than any other organism (it is clear why - because it is their favorite organism - but they should be mindful of the fact that this is to be balanced review).

p. 28, line 10 from bottom:
"In the gree algae…" should read "In the green algae…"

p. 29, para. 2:
Many of the references cited here are inappropriate because they do not deal with bioremediation. Also mention of the role played by S2- in the

fabrication of Cd complexes, for instance vacuolar Cd.PC complexes, should be made in this context. There may not only be upstream but also downstream rate determination(and end-product inhibition) of these processes, as exemplified by the incorporation of S2- into Cd.PC complexes.

Throughout the text, the authors refer to enzymes with Km values for Cd2+ in the micromolar range, yet the likely free concentration of this metal ion in vivo is several orders of magnitude lower because of its reactivity with thiol peptides. This is a problem that we all have to contend with and one that should be stated clearly at the beginning and restated as necessary at other points in the text.

INSTITUTO NACIONAL DE CARDIOLOGÍA
IGNACIO CHAVEZ

Mexico DF 03 August 2004

Dear Professor Brown:

Thank you for your kind letter of 6 June 2004 regarding the manuscript (FEMSRE-04-03-0011) entitled "Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants". We were informed that a major revision of the manuscript addressing the observations made by two reviewers and the editor is required, before the paper may be considered for publication in FEMS Microbiology Reviews. In the enclosed revised manuscript, we have answered all the points raised by the reviewers and the editor. Accordingly, the manuscript was modified as described below; the numbering of the references also changed. We hope that in its present form the manuscript may be found acceptable for publication.

Sincerely

David G. Mendoza-Cózatl
Departamento de Bioquímica
Instituto Nacional de Cardiología
Juan Badiano # 1, Col. Sección XVI, Tlalpan 14080
México, DF. MÉXICO.
Fax: +5255-5573-0926
Email: cozatl@hotmail.com

Response to Editor

To provide a more dynamic and interesting review for the general reader, as requested, we have now critically evaluated, and interpreted, the described information in several places throughout the manuscript. Please see response to reviewer 1, point 1 for specific locations of the modified text. In addition, a general assessment of the data describing the kinetic and genetic properties of the enzymes of the GSH metabolism was added on the section of **Regulation of SAP, Cys and GSH synthesis** on p. 18 and on p. 34 and 35, since the information on the pathway modeling is now no available in the present manuscript version. Please note that the page number is referred to the final PDF version created by Manuscript Central, not that located down in the right corner through the main body of the manuscript

Response to reviewer 1

1. To meet the requested standard for a critical review of the knowledge on sulfur assimilation and GSH metabolism, evaluation and interpretation of data was incorporated in the text at several places. Please see p. 8, 1st paragraph and lines 5-10; p. 10, lines 9-13; p.12, lines 17-19; p. 13, lines 9-12; p. 14, lines 15-17 and 21-24; p. 15, lines 21-22; p. 18 lines 3-4 and last paragraph; p. 19, 1st paragraph and lines 4-9; p. 20, lines 23-24 and 2nd paragraph; p. 21, lines 9-10; p. 23, lines 22-24; p. 25, lines 4-7; p. 26, lines 5-7; p. 28, lines 6-9; p. 29, lines 8-10; and p. 33, 2nd paragraph.

Please note that the page number is referred to the final PDF version, not that located down in the right corner through the main body of the manuscript

2. Recent papers on Cd2+ compartmentation in algae and fungi including results with C. glabrata and S. pombe have been added on p. 6, line 22; p. 19, line 16; p. 25, 2nd paragraph; p. 32, 1st paragraph; p. 31, 1st paragraph; p. 33, last paragraph; and p.34, 1st paragraph.

3. More data on the molecular biology of the systems were included on p. 8, lines 9-10; p. 9, 2nd paragraph; p. 19, lines 16-18; p. 21, lines 2-5 and 22-23; p. 22, lines 1-4; p. 23, lines 4-5; p. 24, 1st paragraph; p. 26, lines 23-24, as requested by the reviewer. In addition, a new sub-section on genetic regulation was added on p. 21 in which most of the molecular biology of the GSH metabolism enzymes under Cd2+ stress is described and analysed.

4. The statement on phytochelatins in C. elegans was deleted from p. 16, 3rd paragraph and corrected on p. 17, 2nd paragraph.

5. The incomplete information on phytochelatin variants has been corrected on p. 17, 1st paragraph.

6. A critical analysis of the identification of phytochelatins with DTNB in different organisms was incorporated on p. 18, lines 11-14, as suggested.

Response to reviewer 2

1. p. 6 last line. The sentence regarding transport of Cd-GSH complexes into the vacuole was modified.

Please note that the page number is referred to the final PDF version, not that located down in the right corner through the main body of the manuscript

2. p. 14 line 13. The word "possesses" is now used.

3. p. 16-18. The information on PCS has been updated.

4. p. 18 lines 7-8. PCs have been detected in S. cerevisiae and N. crassa by mass spectrometry and the result is discussed.

5. p. 25 lines 4-7. We have amended the paragraph regarding the transcriptional control of yeast Cd2+ response. As pointed out by the reviewer, it was misleading as originally stated.

6. p. 26 lines 18-20. The sentence "HMT1 is specific..." was corrected.

7. p. 26 line 20. Li et al 1997 (ref. 158) replaced Ortiz et al 1995 (ref. 22).

8. p. 30-31. Euglena and unicellular algae. Following the reviewer's suggestion, this section was shortened, the paper and sub-section titles slightly changed, and the analysis and interpretation of data toned-down, avoiding unjustified extrapolation from well-described systems.

9. p 32, line 14. "green algae..." was corrected.

10. p. 33, 2nd paragraph. The focus of this paragraph was slightly changed to emphasize.the biochemical characteristics that organisms should have for bioremediation. A statement on the role of S2- in the formation of HMW complexes as a potential control site was added on p. 34, last two lines.

11. Comments on the in vivo free Cd2+ concentration were included on p. 27, lines 8-14, and p. 25, lines 16-21, as requested.

De: <fems-reviews@bham.ac.uk>
Enviado el: Viernes, 17 de Septiembre de 2004 08:36:27 p.m.
Para: cozatl@hotmail.com, davidmendozac@netscape.net
CC: fems-reviews@bham.ac.uk, femsre@fems-microbiology.org, editing@fems-microbiology.org, femsre@fems-microbiology.org
Asunto: FEMSRE-04-03-0011.R1 / Accepted for publication

17-Sep-2004

Dear Dr. David Mendoza-Cozatl,

Manuscript ID: FEMSRE-04-03-0011.R1
Title: Sulfur Assimilation and Glutathione Metabolism Under Cadmium Stress in Yeast, Protists and Plants
Authors: Mendoza-Cozatl, David; Hernández, Andrea; Loza, Herminia; Moreno-Sánchez, Rafael
Submitted to: FEMS Microbiology Reviews
Submitted date: 24-Mar-2004
Revised date: 03-Aug-2004
Accepted date: 17-Sep-2004

I am pleased to be able to tell you that your manuscript detailed above has now been accepted for publication in our journal. I am sorry that it has taken so long from inception to handle your manuscript. Thank you for taking so much care with the revision. I hope you will agree that it has improved substantially.

You will hear from our publisher Elsevier in due course. They will send you a reprint order and copyright transfer form. In principle the electronic files will be used for producing the final publication, but the publisher Elsevier may request a set of high-quality print-outs of your figures for production purposes.

Language editing will be executed by the Editor and the Editorial Coordinator at the FEMS Publications Office. Queries that arise will be directed to you in the e-mailed proofs from the publisher, or when controversial, will first be checked with you.

Sincerely,

Prof. Nigel Brown
Editor
FEMS Microbiology Reviews

P.S. An uncorrected proof of accepted article will on average be published online within five to six weeks after acceptance; please check the Author Gateway of our publisher Elsevier if you have not received e-mailed proofs after six weeks. Note that preparation of the proofs can be delayed if substantial copy-editing is necessary or if a manuscript makes part of a thematic issue.

The authors will have to reply to the proofs, with or without corrections in the proofs, by e-mail WITHIN 48 HOURS.

Del trabajo anterior se puede concluir que el mecanismo de resistencia a Cd^{2+} mediado por tioles (GSH y FQs) se encuentra conservado en tres diferentes reinos de eucariontes (plantae, fungi y protista). Esta amplia distribución en la naturaleza sugiere un alto grado de eficiencia de este mecanismo, resultado de una presión de selección a través del tiempo. Es interesante notar que, independientemente de las diferencias intrínsecas entre los organismos, la resistencia a Cd^{2+} mediada por tioles conserva, a nivel bioquímico, una misma secuencia de eventos: (1) inactivación del metal en el citosol, (2) compartamentalización del Cd^{2+} en algún organelo y (3) formación de complejos de alto peso molecular alrededor de un núcleo de CdS.

Por otro lado, la resistencia a Cd^{2+} no podría explicarse únicamente a nivel bioquímico. Se requiere de una delicada transferencia de información para lograr una activación a diferentes niveles, tanto subcelular como molecular, desde la asimilación de sulfato y su reducción, hasta la síntesis de cisteína, GSH y FQs en diferentes compartimentos, para contender adecuadamente contra el estrés inducido por Cd^{2+}.

Por último, la revisión de los diferentes mecanismos de resistencia a Cd^{2+}, presentes en varios organismos de diferente origen, su similitud y las correspondientes diferencias y adaptaciones, proveen de un marco teórico mucho más completo y adecuado para involucrarse en el estudio de los mecanismos de resistencia a Cd^{2+} en otros organismos tales como *Euglena gracilis*.

Capitulo 3. *Euglena gracilis*

3.1 *Euglena gracilis* como modelo de estudio

E. gracilis es un protista fotosintético con una elevada capacidad de resistencia y acumulación de Cd^{2+}. Su IC_{50} (concentración necesaria para inhibir su crecimiento en un 50%) es de 100 µM, mientras que para microalgas verdes unicelulares como *Chlorella vulgaris*, *C. pyrenoidosa*, *Scenedesmus obliquus* y *S. quadricauda* esta entre 0.03 a 6 µM $CdCl_2$ (Trevors et al., 1996; Devars et al., 1998). Además, *Euglena gracilis* es capaz de acumular cadmio hasta 0.45 % de su peso seco, cuando se cultiva en condiciones fotoheterotróficas con 500 µM $CdCl_2$ y hasta un 1.12 % de su peso seco cuando se cultiva en condiciones heterotróficas con 200 µM $CdCl_2$ (Devars et al., 1998; Avilés et al., 2005); este valor es similar al reportado para plantas *hiperacumuladoras* (Ebbs et al., 2002). Por estas razones, diversos autores han propuesto a este organismo como un candidato ideal para procesos de biorremoción de Cd^{2+} de aguas contaminadas (Bariaud et al., 1985; Navarro et al. 1997, Devars et al., 1998).

Efectos tóxicos del Cd^{2+} en *Euglena gracilis*

Durante más de tres décadas, *E. gracilis* se ha utilizado como modelo para estudiar los efectos tóxicos del Cd^{2+} en células eucariontes. Entre otras características, *Euglena* posee una gran plasticidad metabólica; por ejemplo, la cepa silvestre (fotosintética) puede crecer de manera autótrofa sin necesidad de una fuente adicional de carbono que el CO_2 disuelto en el medio. Si se le cultiva en la oscuridad, se vuelve un organismo completamente heterótrofo, cuyo metabolismo se asemeja más a un protozoario (*Paramecium spp, Tripanosoma spp.*) que a un alga verde. Es interesante notar que *Euglena* puede usar una gran variedad de fuentes de carbono, tales como glucosa, lactato, etanol, succinato y piruvato, entre otros (Buetow, 1989), y además de poder

sobrevivir en condiciones de baja tensión de oxígeno por periodos relativamente largos (Buetow, 1989). Como una tercera posibilidad, *Euglena* puede crecer fotoheterotróficamente (Buetow, 1989). Estas características hacen a *Euglena* un modelo aún más atractivo para estudiar la toxicidad del Cd^{2+}.

Por otro lado, esta misma plasticidad metabólica complica la comparación directa de resultados entre grupos de investigación, pues aumenta las variables a considerar. Por ejemplo, varios autores cultivan a *E. gracilis* en condiciones de iluminación constante, una condición muy alejada de lo fisiológico, y encuentran una LC_{50} (dosis letal media) para Cd^{2+} de 1.36 µM (Einicker-Lamas et al., 2003), mientras que en *Euglena* cultivada con un foto-periodo de 12 h luz/12 h oscuridad se determinó una IC_{50} de 100 µM, la cual sugiere una LC_{50} >> 100 µM (Devars et al., 1998). La fuente de carbono es otra variable a considerar. Cuando se cultiva *E. gracilis* en la oscuridad, se observa una mayor respiración celular y actividad de la citocromo c oxidasa utilizando lactato como única fuente de carbono comparado con un medio con glutamato-malato, tanto en células control como en células expuestas a Cd^{2+} (Navarro et al., 1997). La tabla 3.1 muestra los principales efectos del Cd^{2+} en *Euglena gracilis,* especificando las condiciones de cultivo y la concentración de Cd^{2+} usadas en cada trabajo. Hay que notar que la toxicidad del Cd^{2+} esta estrechamente relacionada con la concentración usada y sus principales efectos son : inhibición del crecimiento, de la fotosíntesis, de la respiración celular, de la división celular, multinucleación, aumento en el contenido de DNA, cambios en la estructura del cloroplasto y de la mitocondria, acumulación de paramilo (polímero de glucosa con enlaces glucosídicos β 1-3), inducción de proteínas de estrés (HSP), aumento en los tioles solubles en ácido (Cys, γ-EC, GSH, FQs) y generación de especies reactivas de oxígeno (Tabla 3.1).

Tabla 3.1 Efectos del Cd^{2+} en *Euglena gracilis*

Concentraciónde Cd^{2+}	Condiciones de cultivo[a]	Efectos	Referencia
50 µM	G+M, oscuridad	Inhibición del crecimiento solo en un medio deficiente de Zinc (10 nM), acumulación de paramilo, multinucleación y aumento de 3 a 4 veces en el contenido de DNA	Falchuck et al., 1975
20-88 µM	Fotoperiodo	Inhibición del crecimiento; Cd^{2+} unido mayoritariamente a un compuesto de más de 100 kDa	Albergoni et al., 1980
50 µM	Lac, luz 24h	Incremento en el contenido de DNA, inhibición del ciclo celular en fase G1	Bonaly et al., 1980
0.1 µM	luz 24h	Del 1er al 4to día de cultivo, estimulación de la fotosíntesis y respiración; después del 4to día, inhibición de la fotosíntesis y respiración	De Filippis et al., 1981
0.5 mM	Lac, luz 24h	Una cepa resistente de *E. gracilis* pre-adaptada a Cd^{2+} (0.5 mM) capta menos Cd^{2+} que la cepa silvestre	Bariaud et al., 1985
50 µM	Lac, luz 24h	Cambios en la estructura del cloroplasto y mitocondrias	Duret et al., 1986
20-88 µM	Fotoperiodo	Aumento en el contenido de tioles solubles en ácido incluyendo Cys y GSH	Coppellotti, 1989
0.5-2 mM	Lac, obscuridad	Inducción de proteínas de estrés: hsp90, hsp70, hsp55 y hsp40	Barque et al., 1996
50-500 µM	G+M, fotoperiodo	Inhibición de la respiración y de producción de oxígeno, aumento de dos veces en la concentración de clorofila	Navarro et al., 1997
50-500 µM	G+M, fotoperiodo	Disminución en el crecimiento, incremento en la respiración e inhibición de la fotosíntesis	Devars et al., 1998
50-500 µM	G+M, fotoperiodo	Generación de especies reactivas de oxígeno y fragmentación del DNA nuclear	Watanabe et al., 2003
1.36 µM	luz 24h	Participación de una proteína similar a una P-glicoproteína que contribuye a la resistencia a Cd^{2+}; su inhibición hace a *Euglena* más sensibles al Cd^{2+}	Einicker-Lamas et al., 2003

[a] Medio de cultivo: G+M, glutamato-malato; Lac, lactato. Fotoperiodo, 12h luz/12h obs; obscuridad, obscuridad constante; luz 24h, iluminación constante. Donde se omite el medio de cultivo, este no fue mencionado por los autores o lo refieren como "condiciones estándar".

3.2 Mecanismos de resistencia a Cd^{2+} descritos en *Euglena gracilis*

Hacia el año 2001, se estableció que *Euglena gracilis* no sintetizaba metalotioneínas, proteínas de bajo peso molecular (<40 kDa) ricas en cisteínas, pero que sí sintetizaba fitoquelatinas (FQs, ver capitulo 2) (Bariaud et al., 1985; Gekeler et al., 1988). Además, se había determinado que la asimilación y reducción de sulfato estaba relacionado con el mecanismo de resistencia, debido a que los tioles solubles en ácido, incluyendo la cisteína (Cys) y el glutatión (GSH), se incrementaban debido a la exposición a Cd^{2+} (Tabla 3.1; Coppellotti, 1989; Devars, 1998). Se sabía además que la mayor parte del Cd^{2+} estaba unido a moléculas de alto peso molecular (> 100 Da), pero la naturaleza química de estas no había sido determinada (Albergoni, 1980). En 1987, se caracterizó un compuesto que unía fuertemente Cd^{2+} y que contenía sulfuro en una relación Cd^{2+}/S^{2-} = 0.8, pero tampoco se determinó su composición, secuencia de aminoácidos o peso molecular (Weber et al., 1987). Por otro lado, solo el 10 % del Cd^{2+} captado por la célula se localizaba en la fracción soluble (citosol), por lo que se presumía la compartamentalización del Cd^{2+} (Bariaud et al., 1985).

Debido a que *E. gracilis* carece de una vacuola tipo planta que abarque más del 80 % del volumen intracelular, no era posible proponer una analogía directa con el mecanismo de resistencia mediado por tioles en plantas y *Schizosaccaromyces pombe* (Buetow, 1989; ver capitulo 2, Mendoza-Cózatl et al., 2005). Así, El primer paso que dimos para determinar el mecanismo de resistencia a Cd^{2+} en *Euglena* fue determinar la distribución intracelular, tanto de Cd^{2+} como de tioles, y establecer si su compartamentalización podía relacionarse con el mecanismo de resistencia a Cd^{2+} en *Euglena*.

Capitulo 4. Acumulación de Cd^{2+} en el cloroplasto de *Euglena gracilis*

pag 47-54 Physiologia Plantarum 115:276-283

PHYSIOLOGIA PLANTARUM 115: 276–283. 2002
Printed in Denmark – all rights reserved

ISSN 0031-9317

Cadmium accumulation in the chloroplast of *Euglena gracilis*

David Mendoza-Cozatl[a], Silvia Devars[a], Herminia Loza-Tavera[b] and Rafael Moreno-Sánchez[a,*]

[a] *Departamento de Bioquímica, Instituto Nacional de Cardiología, D.F. 14080, Mexico*
[b] *Departamento de Bioquímica, Facultad de Química, UNAM, D.F. 04510, Mexico*
**Corresponding author, e-mail: morenosanchez@hotmail.com*

Received 29 August 2001; revised 29 November 2001

Intracellular distribution of Cd, cysteine, glutathione, and Cd-induced thiol peptides in *Euglena gracilis* cultured under photoheterotrophic conditions was studied. After 3 days of culture with 0.2 m*M* $CdCl_2$, 62% of the Cd accumulated by cells was equally distributed between the cytosolic and chloroplastic fractions. However, after 8 days, metal content increased in the crude chloroplastic fraction to 40% of total and decreased to 19% in the cytosol; in Percoll-purified chloroplasts the estimated content of Cd raised to 62%. Accumulation of Cd in chloroplasts could be mediated by a transporter of free Cd^{2+}, since uptake of added $CdCl_2$ in isolated chloroplasts exhibited a hyperbolic type of kinetics with a K_m of 57 μ*M* and V_{max} of 3.7 nmol (mg protein)$^{-1}$ min^{-1}. The contents of cysteine and glutathione markedly increased in both chloroplasts (7–19 times) and cytosol (4–9 times) by exposure to Cd^{2+}, although they were always higher in the cytosol. Thiol-containing peptides induced by Cd were mainly located in the cytosol after 3 days, and in the chloroplasts after 8 days of culture. The data suggested that Cd was compartmentalized into chloroplasts in a process that may involve the transport of free Cd and the participation of thiol-peptides.

Introduction

Toxicity of Cd in the physiology of diverse organisms including man (Vallee and Ulmer 1972), algae (Reed and Gadd 1989), fungi and bacteria (Trevors et al. 1986) is well documented. In *Euglena gracilis*, toxic effects of Cd include inhibition of growth, motility, phototaxis and photosynthesis (De Filippis et al. 1981, Stallwitz and Häder 1993, De Filippis and Pallaghy 1994).

Resistance to cadmium in higher plants and yeast has been related to the induction of phytochelatin (glutathione-derived polymers) synthesis, which chelate the metal in the cytosol and transport it into the vacuole (Zenk 1996). In yeast, a cadmium bis-glutathionate complex can also be transported into the vacuole (Li et al. 1997). Compartmentation of heavy metals has also been described in algae. *Scenedesmus* preferentially accumulates copper in the nucleus and vacuole (Silverberg et al. 1976); *Porphyra* and *Fucus* accumulate Cd in the nucleus (Lignell et al. 1982) and *Chlamydomonas* in the chloroplast (Nagel et al. 1996). In *Euglena*, reports on the subcellular distribution of heavy metals are scarce. Bariaud et al. (1985) detected only 10% of Cd accumulated by *Euglena* in the cytosol. Since a typical vacuole is absent in *Euglena* (Briand and Calvayrac 1980, Bertaux et al. 1989), compartmentalization of Cd into other organelles may be expected. Phytochelatin synthesis and enhanced glutathione content induced by Cd has also been reported in this microorganism (Gekeler et al. 1988, Coppellotti 1989).

An enhanced accumulation of Cd in *Euglena gracilis* grown in the dark was obtained by changing the carbon source in the culture medium or by pre-treating cells with 1.5 μ*M* $HgCl_2$ (Navarro et al. 1997). Cells grown under light/dark cycles showed a 2 to 3-fold greater accumulation of Cd than dark-grown cells (Navarro et al. 1997). The ability of photosynthetic cells to accumulate Cd was also raised by pre-treatment with low mercury concentrations (Devars et al. 1998). The elucidation of the biochemical strategies that allow resistant cells to accumulate heavy metals is of interest for bioremediation purposes. Therefore, to advance the understanding of the biochemical mechanisms of Cd resistance, in this work the subcellular distribution of Cd, cysteine, glutathione, and thiol-containing peptides was determined in *Euglena gracilis* cultured with Cd under photoheterotrophic conditions.

Materials and methods

Cell culture and growth conditions

Axenic cultures of *E. gracilis* Klebs (strain Z) were grown under photoheterotrophic conditions with cycles of 12 h

of fluorescent white light (60–70 μmol quanta m^{-2} s^{-1}) and 12 h dark at 25°C, in a medium that contained glutamate and malate as carbon source (Devars et al. 1998). Cadmium was added from a stock solution (25 m*M*) of $CdCl_2$, stored at 4°C. Cultures were initiated with an inoculum of 2×10^5 cells ml^{-1}; cells were previously grown under light-dark cycles for at least 15 days, and transferred every 5 days, during the exponential phase, to fresh medium. Cells were counted in a Neubauer chamber after appropriate dilution and immobilization with HCl (2.2% v/v). Cell viability was determined after incubation at room temperature with 0.025% (w/v) trypan blue for 10 min. After harvesting by centrifugation at 1000 *g* for 10 min at 4°C, cells were washed once with 10–15 volumes of SHE buffer (250 m*M* sucrose, 10 m*M* HEPES (4-(2-hydroxyethyl) piperazine-1-ethane sulphonic acid), and 1 m*M* EGTA (ethylene glycol-bis(β-amino-ethyl ether)N,N,N′,N′-tetraacetic acid), pH7.2). The cell pellet was resuspended in a small volume of SHE buffer and an aliquot of 10×10^6 cells was stored at −70°C until the metal content was determined.

Subcellular fractionation

Cells were subjected to sonication as described previously (Moreno-Sánchez and Raya 1987) in a Branson 450 sonifier until 70–80% of cells were disrupted. The sonicated suspension was diluted to 20 ml with SHE buffer and centrifuged at 50 *g* for 5 min; nuclei and undisrupted cells were sedimented in this fraction. The supernatant was centrifuged at 2000 *g* for 10 min to obtain a crude chloroplastic fraction. The supernatant was then centrifuged at 13 000 *g* for 10 min to obtain the mitochondrial fraction. The last supernatant was centrifuged at 225 000 *g* for 45 min to obtain the cytosolic fraction in the supernatant and the microsomal fraction in the pellet. All the centrifugation steps were carried out at 4°C.

Intact chloroplasts were also purified according to the method of Price et al. (1987) with some modifications. Approximately 1 l of culture was incubated in the dark 15 h prior to isolation. An aliquot of 1×10^9 cells was disrupted by sonication in an isolation medium (0.33 *M* sorbitol, 1 m*M* $Na_4P_2O_2$, 50 m*M* HEPES, 2 m*M* EDTA, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, pH6.8). The homogenate was centrifuged at 2000 *g* for 10 min; the pellet was resuspended in fresh isolation medium and centrifuged at 6500 *g* for 20 min in a discontinuous gradient of 40–80% (v/v) Percoll, dissolved in isolation medium. The heavy band at the interface collected between 40 and 80% Percoll was washed once with isolation medium and considered as the intact, purified fraction of chloroplasts.

To assess the chloroplast integrity, the rate of photophosphorylation was measured by following the consumption of H^+ with a glass pH electrode (Nishimura et al. 1962). Chloroplasts (1–3 mg protein) were incubated in a medium that contained 0.33 *M* sorbitol, 1 m*M* $Na_4P_2O_7$, 1 m*M* HEPES, 1 m*M* ADP, 1 m*M* $MgCl_2$, 5 m*M* KH_2PO_4, with an initial pH of 7.5. Photophosphorylation was initiated by illumination with saturating white light (1000 μmol quanta m^{-2} s^{-1}); this reaction was inhibited by 10 μ*M* CCCP or 20 μ*M* DCMU (3-[3,4-dichlorophenyl]-1,1-dimethyl urea).

Determination of protein and chlorophyll content

Protein concentration was determined by the biuret method using bovine serum albumin as standard. To discard cadmium interference, protein was also measured by the Bradford (1976) and Murphy and Kies (1960) methods. Turbidity from paramylum was eliminated by centrifugation of the samples at 1360 *g* for 10 min. The content of chlorophyll was determined spectrophotometrically in 80% acetone extracts by the method of Arnon (1949).

Marker enzymes

Aliquots of the initial sonicated suspension and of each subcellular fraction were stored at −70°C until use. The activity of the marker enzymes, determined in the freshly obtained fractions or in the frozen and thawed samples, gave similar results. The different cell fractions were identified by measuring specific enzymatic activities or DNA and chlorophyll content. DNA was quantified by measuring the fluorescence of DAPI (4,6-diamidine-2-phenylindole) by the method of Brunk et al. (1997).

Chloroplastic fraction. The activity of photosystem II was measured at 25°C by following the oxygen consumption with a Clark-type oxygen electrode (YSI model 53; Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA), using water as electron donor and methylviologen as acceptor in 0.1 *M* sucrose, 5 m*M* $MgCl_2$, 15 m*M* HEPES, 10 m*M* KCl, pH8.0. The reaction was started by illumination with saturating white light (1000 μmol quanta $m^{-2} \cdot s^{-1}$); the activity resistant to DCMU was subtracted (Allen and Holmes 1986).

The activities of cytochrome c oxidase (mitochondrial fraction), glucose-6-phosphatase (microsomal fraction) and acid phosphatase (lysosomal fraction) were determined as described elsewhere (Graham 1993, Devars et al. 1998).

Cd accumulation

Cadmium was determined in thawed aliquots of *Euglena* cells washed with SHE buffer and in thawed aliquots of each subcellular fraction. Aliquots of 0.05–1 ml from cell extracts and fractions were digested by boiling in 0.5 ml of 99% (v/v) H_2SO_4 + 2.5 ml of 70% (v/v) HNO_3 for 2 h in a block heater. The content of Cd in the digested clear samples was determined in an atomic absorption spectrophotometer (SpectrAA 640, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).

Cd^{2+} uptake

Percoll-purified chloroplasts, isolated from cells grown in the presence of either 0.05 or 0.2 m*M* $CdCl_2$ for 8

days, were incubated at 25°C in a medium that contained 0.33 *M* sorbitol, 1 m*M* HEPES, pH 7.5, and different concentrations of $^{109}CdCl_2$ (specific activity 5550–7400 Bq μmol^{-1}). At pre-determined times, 0.5 ml aliquots were withdrawn, filtered through nitrocellulose filters (0.45 µm pore diameter, Millipore Corporation, Bedford, MA, USA) previously rinsed with 10 m*M* $CdCl_2$, and washed twice with 5 ml of ice-cold 10 m*M* $CdCl_2$. Radioactivity in the filters was measured in a liquid scintillation counter.

Content of cysteine, glutathione, and thiol-containing peptides

Aliquots of the fractions (10 mg protein) were diluted to a final volume of 2 ml with KME buffer (120 m*M* KCl, 20 MOPS, 2 m*M* EGTA, pH 7.2), extracted with 3% cold perchloric acid, mixed, and centrifuged at 2250 *g* for 5 min. Aliquots of supernatant were analysed by high performance liquid chromatography (HPLC), in a C-18 Waters Nova-Pak column (4.6 mm × 150 mm; Spherisorb S5 OD52) equilibrated with 0.05% H_3PO_4 and eluted with a linear 1–20% acetonitrile gradient during 40 min. Sulfhydryl groups from cysteine, glutathione, and thiol-peptides were detected by post-column derivatization with 0.1 m*M* DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]), recording absorbance at 412 nm (Grill et al. 1991).

Results

Cell growth, metabolite content, and enzyme activities

Euglena gracilis strain Z initiated the logarithmic phase of growth after 3 days of culture under photoheterotrophic conditions; the early stationary phase was reached around the 7th day (data not shown). The addition of $CdCl_2$ slowed down the growth rate and severely decreased the cellular density reached in the stationary phase (Table 1); however, growth was not abolished in the presence of 0.2 m*M* $CdCl_2$. The cell viability was always higher than 90%, even in the presence of Cd. The chlorophyll content was not significantly affected by Cd^{2+} exposure. The protein content, measured by the biuret method, increased 2–2.5 times (Table 1), and by the Bradford and the Murphy and Kies assays, 5.1 and 7.1 times, respectively. The absorbance spectrum of chlorophyll and the biuret assay for proteins were not altered by the addition of 1 m*M* $CdCl_2$ (data not shown).

The HPLC analysis of the cell extracts revealed a pronounced increase in the contents of cysteine and glutathione induced by exposure to Cd^{2+} (Table 1). In addition, Cd also prompted the formation of acid-soluble, thiol-containing peptides of a size greater than glutathione, presumably phytochelatins (Gekeler et al. 1988, Rauser 1990); these peptides were not detected in extracts from cells grown in the absence of Cd^{2+} (Table 1; Fig. 3A).

The DNA content and the activity of the photosystem II were markedly increased by Cd, whereas the TMPD oxidase activity was diminished (Table 1).

Identification of subcellular fractions

The activity of the donor side of photosystem II was detected in all fractions except in the cytosol, but it was highest in the 2000 *g* pellet. The highest content of chlorophyll was also found in the 2000 *g* pellet, which indicated that the main fraction of chloroplasts was recovered in this centrifugation step. Further purification of this fraction resulted in a significant increase in the rate of photophosphorylation, from 55 (n = 2) before the Percoll step, to 220 ± 94 nmol ATP (mg protein)$^{-1}$ min^{-1} (mean ± SD; n = 5). Percoll-purified chloroplasts from cells grown with 0.05 m*M* Cd^{2+} exhibited a photophosphorylation rate 3-fold lower than the control (72 ± 17 nmol ATP (mg protein)$^{-1}$ min^{-1}; n = 3).

The TMPD oxidase activity was distributed among all the different cell fractions (data not shown). This activity was severely depressed by Cd in both whole cells (Table 1) and in the different fractions. The crude chloroplastic fraction exhibited a TMPD oxidase activity almost as high as that found in the mitochondrial fraction. However, this activity was 92% eliminated after the Percoll centrifugation step (data not shown). Suzuki et al. (1987) reported no contamination by the cytosolic fraction and only 5% by mitochondria in chloroplasts isolated with a similar purification procedure.

Table 1. Effect of Cd^{2+} on growth, metabolite content, and enzyme activities of photoheterotrophic grown *Euglena*. Means ± SD (n). The thiol-containing peptides represent the sum of all thiol-containing compounds induced by cadmium with retention times in the HPLC chromatogram greater than glutathione (see Fig. 3). NM, not measured.

Time of Culture	3 days	8 days	3 days (0.2 m*M* Cd)	8 days (0.2 m*M* Cd)
Growth × 10^6 cells ml^{-1}	2.1 ± 0.2 (3)	5.3 ± 0.9 (5)	0.2 ± 0.1 (3)	0.9 ± 0.1 (5)
Cd Content nmol/10^7 cells	0	0	194 ± 42 (3)	249 ± 30 (4)
Protein (mg/10^7 cells)	5.5 ± 1.5 (3)	4.0 ± 0.7 (5)	9.8 ± 1.9 (3)	10.4 ± 0.8 (4)
Chlorophyll (µg/10^7 cells)	45 ± 16 (3)	42 ± 10 (5)	40 ± 9 (3)	55 ± 15 (4)
Cys (nmol/10^7 cells)	NM	5 ± 2 (3)	45 ± 15 (3)	36 ± 10 (3)
GSH (nmol/10^7 cells)	7.5 (1)	7 ± 2 (4)	63 ± 25 (3)	84 ± 30 (4)
Thiol-containing peptides (nmol SH/10^7 cells)	0 (3)	0 (4)	25 ± 9 (3)	33 ± 10 (4)
DNA (µg/10^7 cells)	NM	10.9 (2)	NM	32 ± 5 (3)
PSII ng atoms oxygen ($min \times 10^7$ cells)$^{-1}$	NM	116 ± 10 (4)	NM	480 ± 36 (4)
TMPD oxidase ng atoms oxygen ($min \times 10^7$ cells)$^{-1}$	NM	75 ± 16 (4)	NM	36 ± 5 (4)

Intracellular distribution of Cd

After 3 days of culture, 62% of accumulated cadmium was equally distributed between the chloroplastic and the cytosolic fractions (Fig. 1A); the remaining accumulated Cd was located in the nuclei (24%), mitochondria (8%), and microsomal (6%) fractions (data not shown). To establish whether sonication of cells disturbed the intracellular distribution of Cd, cells were grown without metal and mixed, prior to sonication, with 270 nmol Cd/10^7 cells, which was an amount of metal similar to that accumulated by cells. Under these conditions, and in contrast to data shown in Fig. 1A, the cytosol retained the largest amount of Cd (> 88%; data not shown).

Interestingly, after 8 days of culture the content of Cd only increased in the chloroplast, amounting up to 40 ± 5% of total accumulated Cd, and diminished in the cytosol to 19 ± 5% (Fig. 1A). Since this crude chloroplastic fraction was heavily contaminated with mitochondria, it was decided to further purify the chloroplastic fraction using a Percoll gradient. The Percoll-purified fraction showed negligible activity of the mitochondrial marker enzyme. The metal to chlorophyll ratio in the crude chloroplastic fraction was 1.6 ± 0.42 nmol Cd (μg Chl)$^{-1}$ (mean of 4 experiments ± SD). This ratio increased to 3.15 ± 1.1 nmol Cd (μg Chl)$^{-1}$ (n = 4) in Percoll-purified chloroplasts. Thus, using this ratio and the cellular contents of Cd and chloroplyll (Table 1), 62 ± 12% of total (n = 4) was estimated for Cd internalized into chloroplasts instead of 40%. Therefore, the increased metal uptake from the 3rd to the 8th day was mainly due to an increased compartmentalization into the chloroplast. Similarly, in a cell wall-deficient strain of *Chlamydomonas* only 10% of incorporated ^{109}Cd was found in the cytosol and > 50% in Percoll-purified chloroplasts (Nagel et al. 1996).

The rate of Cd^{2+} uptake at 25°C in intact Percoll-purified chloroplasts, isolated from cells grown for 8 days with 0.05 m*M* $CdCl_2$, followed a Michaelis-Menten (hyperbolic) type of kinetics, as revealed by the non-linear (Fig. 2) and the linear curve fitting (Fig. 2, insert) of the experimental data. The estimated Michaelis-Menten constant (K_m) was 57 ± 13 μ*M* and the V_{max} was 3.7 ± 0.5 nmol (mg protein)$^{-1}$ min^{-1} (mean ± SEM, n = 3). At

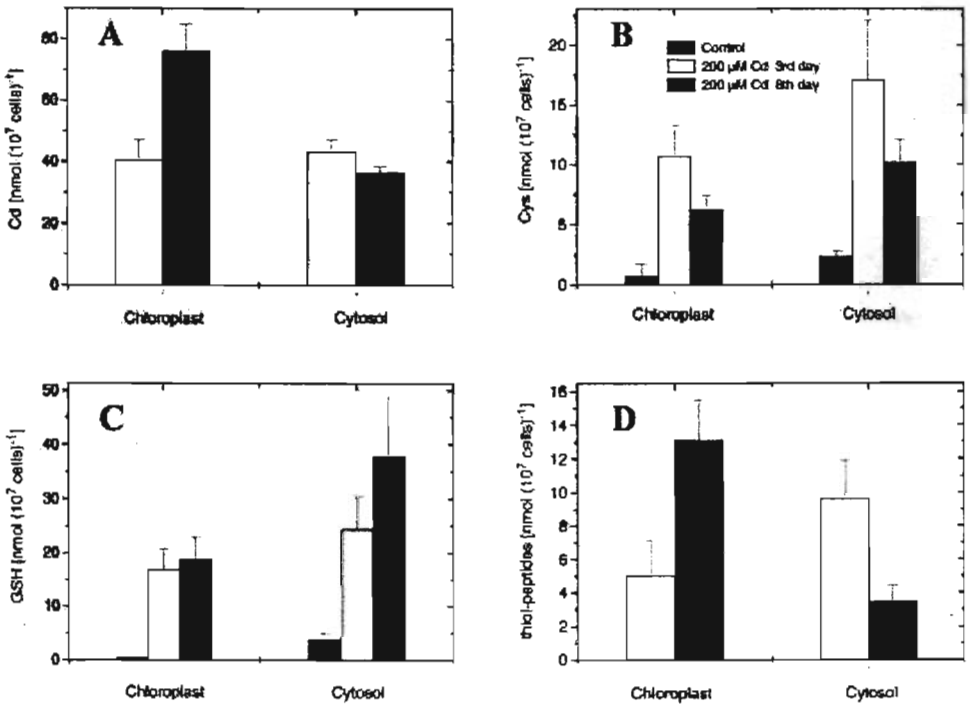


Fig. 1. Distribution of Cd, cysteine, glutathione, and thiol-containing peptides in subcellular fractions of *Euglena gracilis*. Cells were harvested after 3 or 8 days of culture with or without 0.2 m*M* $CdCl_2$. Cell fractionation and determination of the contents of Cd (A), cysteine (B), glutathione (C), and thiol-peptides (D) were made as described under Methods. The percentage of non-recovered metabolites after fractionation was 43 ± 11% (n = 22; mean ± SD). The total amount of metabolites was normalized to 107 cells and the 'chloroplast' plus 'cytosol' fractions amounted up 70% of total. Values are the mean ± SD of 3–4 different preparations.

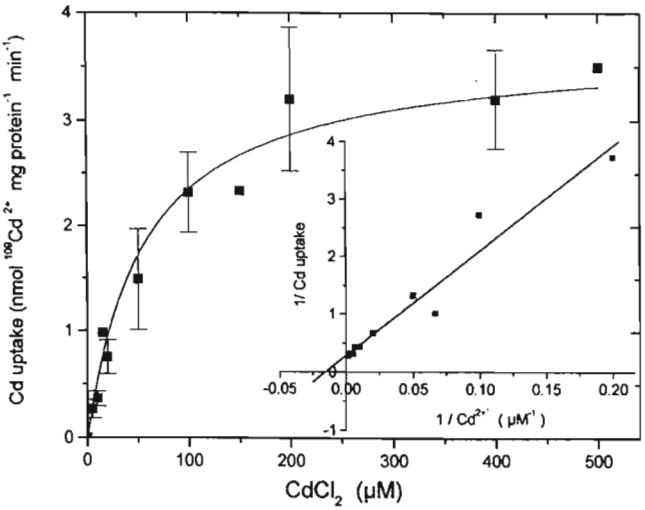


Fig. 2. Kinetics of the Cd^{2+} uptake in *Euglena* chloroplasts. Chloroplasts were isolated using a Percoll gradient centrifugation procedure as described under Methods, from cells grown for 8 days in the presence of 0.05 m*M* $CdCl_2$. Purified chloroplasts (1 mg protein) were added to 2.5 ml of the sorbitol medium, described under Methods, to start the reaction; the medium also contained the indicated concentrations of $^{109}CdCl_2$. An aliquot was filtered every 5 min during 30 min. To discard unspecific binding, the rate of Cd^{2+} uptake was determined from the slope of the plot Cd retained in the filter versus time. Such a plot was linear for at least 30 min. The data shown represent the mean ± SD of 3 different preparations. The solid lines represent the best fit to the Michaelis-Menten and the Lineweaver-Burk (insert) equations.

$CdCl_2$ concentrations of 0.4 m*M* or higher, an apparent non-saturable component was also observed (not shown). A similar kinetic pattern was obtained for Cd^{2+} uptake in chloroplasts purified from cells grown with 0.2 m*M* $CdCl_2$. Incubation at 4°C of chloroplasts, isolated from control and Cd-exposed cells, only partially (37 ± 9 and 10 ± 9%; n = 3, respectively) inhibited Cd^{2+} uptake assayed at 0.05 m*M* $CdCl_2$.

Intracellular distribution of cysteine, glutathione and thiol-peptides

The HPLC analysis of the Percoll-purified chloroplasts (Fig. 3), and of the other cell fractions (data not shown), showed that the increase in the cellular contents of cysteine, glutathione, and thiol-containing peptides, induced by exposure to Cd^{2+} (Table 1), were largely located in the chloroplastic and cytosolic fractions (Fig. 1B-D). Remarkably, the cysteine and glutathione contents were enhanced 7–11 and 17–19 times in chloroplasts, respectively, whereas in the cytosol their contents were elevated 4–7 and 6–9 times (Fig. 1B,C). The levels of cysteine and glutathione (Fig. 1B,C and Fig. 3) at the 8th day were not significantly different from those at the 3rd day of culture.

In agreement with data obtained with the cellular extracts (Table 1), thiol-containing peptides were not detected in the cytosolic (not shown) or chloroplastic fractions (Fig. 3) derived from cells grown in the absence of Cd^{2+}. After 3 days of culture with Cd, the thiol-peptides were mainly (56% of total) located in the cytosol. However, by the 8th day of culture, about 57% of the thiol-peptides were now located in the chloroplasts and 29% in the cytosol, correlating with the Cd distribution pattern (cf. Fig. 1A,D). The contents of Cd and thiol compounds, in Percoll-purified chloroplasts isolated from cells grown with 0.2 m*M* $CdCl_2$ for 8 days, were (per mg protein): 26 ± 6 nmol Cd (n = 6), 3.1 ± 2 nmol cysteine (n = 3), 7.6 ± 1.9 nmol glutathione (n = 3), and 6.6 ± 2.5 nmol thiol-peptides (n = 3).

Discussion

Little is known about the subcellular distribution of Cd and the metabolites induced in response to Cd stress in vacuole-lacking cells. We previously demonstrated that *Euglena gracilis* Z strain grown photoheterotrophically was able to accumulate significant amounts of Cd (Devars et al., 1998). Thus in this work we determined the intracellular distribution of Cd and some other metabolites related to the Cd^{2+} response in this microorganism. The accumulation of Cd by the whole cells (cf. Table 1) was similar to previous values reported by Coppellotti (1989) and our group (Devars et al. 1998).

Cd^{2+} exposure slowed down cell growth but did not affect cell viability. The increase in the protein content indicated that, despite the reduced ATP supply from mitochondria (diminished TMPD oxidase) and chloroplasts (diminished photophosphorylation), the protein synthesis machinery was active in the presence of the heavy metal. Inhibition by Cd of the protein degradative processes, which may require ATP, may also be involved in the increase of the cellular protein content.

Compartmentalization of Cd in the vacuoles of yeast and plants has been proposed as a protective mechanism against the damaging effects of this metal (Ortiz et al. 1992, Zenk 1996, Li et al. 1997). Cd accumulation in nuclei, mitochondria and chloroplasts has also been ob-

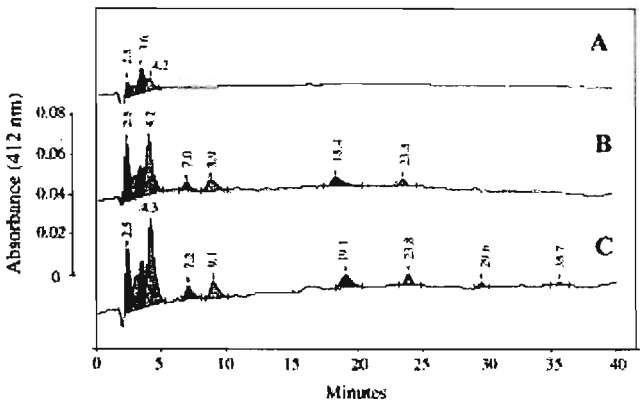


Fig. 3. HPLC profiles of DTNB-reacting compounds from Percoll-purified chloroplasts. Chloroplasts isolated from 8 day-cultured, control cells (A), and 3 (B) and 8 day-cultured cells with 0.2 m*M* $CdCl_2$ (C) were acid-extracted, filtered, separated and detected by reaction with DTNB as described under Methods. Identical amounts of protein (0.55 mg) were analysed under the three conditions. Peaks with retention times of 2.5 and 4.3 min correspond to cysteine and glutathione, respectively. Peaks developed at longer times correspond to thiol-containing peptides induced by Cd. The compounds detected by DTNB reaction at 412 nm after glutathione also showed an absorbance peak at 220 nm, wavelength at which the peptide bond has an absorbance maximum (data not shown). Cd-binding peptides, isolated from *Euglena* grown in the presence of 0.05 m*M* $CdCl_2$ for 8 days as described by Grill et al. (1991), showed an absorbance peak at 254 nm indicative of Cd-thiolate coordinates. These peptides exhibited an identical HPLC profile to that of Fig. 3C.

served, although this compartmentalization seems not to be a defense mechanism since detrimental effects are observed in the structure and metabolism of these organelles (Falchuk et al. 1975a,1975b, Lignell et al. 1982, Nagel et al. 1996). A higher DNA content in the Cd-exposed *Euglena* cells (Table 1) suggests that DNA synthesis is active but cell division is arrested, raising the possibility of a blocking of the cell cycle. A similar increase in the DNA content was described by Falchuk et al. (1975a) in *Euglena* grown with 10 μ*M* Cd^{2+}. Multinucleated cells, as a result of inhibition of cytokinesis, fragmented endosomes, diffuse chromatin and increased DNA contents, have been observed in *Euglena* after Cd^{2+} exposure, indicating alterations in the structure or composition of intranuclear components (Falchuk et al., 1975b).

Chloroplast compartmentalization of Cd accompanied by a strong inhibitory effect on photosynthesis has been observed in *Chlamydomonas reinhardtii* (Nagel et al. 1996). In the present work we demonstrate that Cd is also accumulated in *Euglena* chloroplasts, which leads to inhibition of photophosphorylation. Inhibition of photosynthesis by Cd in intact *Euglena* cells has also been described (Devars et al. 1998). The stimulation of the donor side of the photosystem II by Cd may be the result of uncoupling between electron transport and H^+ pumping.

Several metabolic events related to GSH metabolism are involved in the cellular response to Cd in many organisms. In *Euglena*, cysteine and glutathione increased significantly after 3 days of Cd^{2+} exposure. Since the levels of these metabolites did not substantially change after further incubation with Cd^{2+}, the enzymes involved in the synthesis of these compounds must have been induced/activated by the initial exposure to the metal. The contents of cysteine and glutathione in control cells were similar to those reported by Coppellotti (1989); this author also described a 6-fold increase in the cysteine and glutathione contents in *Euglena* grown in the presence of 44.5 μ*M* $CdCl_2$. Increases in the cysteine concentration, concomitant with an increased expression of the enzymes for sulphur assimilation, ATP sulphurylase and adenylylsulphate (APS) reductase, have also been observed after Cd^{2+} exposure in *Brassica juncea* (Schäfer et al. 1998). This indicates that the flux through this pathway is stimulated in response to Cd.

In higher plants and some algae, the enzymes for the synthesis of cysteine and glutathione have been located in the cytosol (Nussbaum et al. 1988, Lunn et al. 1990) and in chloroplasts (Hell and Bergmann 1990). In *B. juncea*, protein sequences from cDNA clones encoding *O*-acetyl serine (thiol) lyase, γ-glutamylcysteine synthetase and glutathione synthetase, which exhibit putative mitochondrial targeting sequences, have been isolated from Cd^{2+} exposed plants (Schäfer et al. 1998). These observations indicate that a cysteine biosynthetic pathway may be present in mitochondria. In purified mitochondria from proplastid-lacking *Euglena* mutants, the enzyme activities for sulphur activation, ATP sulphurylase and APS kinase, have been identified (Saidha et al. 1988). In contrast, in *Euglena* purified chloroplasts these enzyme activities were not detected (Saidha et al. 1988). However, for chloroplasts, it is not known whether the other enzymes for cysteine biosynthesis are present, whether this organelle imports this amino acid from the cytosol for protein and glutathione synthesis, or whether the expression of nuclear-encoded chloroplast enzymes for this biosynthetic pathway is induced by Cd.

PC synthesis is an evolutionarily well-conserved mechanism proposed to deal with heavy metal toxicity in plants, yeast and algae (Gekeler et al. 1988, Clemens

et al. 1999, Cobbett 2000). Cd-induction of PC synthesis in *Euglena* was originally described by Gekeler et al. (1988), but no subcellular distribution was analysed. In *Chlamydomonas reinhardtii*, thiol-containing peptides increased in cytosol and chloroplasts after Cd^{2+} exposure (Nagel et al. 1996). In our work, Cd-induced thiol-peptides, presumably PCs, are present in both the cytosol and chloroplasts since the 3rd day of Cd^{2+} exposure. The total content of the thiol-containing peptides was within the range reported by Gekeler et al. (1988) for *Euglena* grown with 20 μM $Cd(NO_3)_2$ and harvested during the logarithmic phase.

The identity of the thiol-peptides induced by Cd was not elucidated, but the compounds with retention times of 18 and 23 min (Fig. 3B,C) probably correspond to phytochelatins-2 and 3 (PC-2 and PC-3) (Al-Lahham et al. 1999). The other two compounds with longer retention times, synthesized by *Euglena* after 8 days of culture with 0.2 mM Cd^{2+} (Fig. 3C), might also be phytochelatins although longer than PC-4 since no thiols appeared around 25 min (Al-Lahham et al. 1999). The identity of the compounds with retention times of 3.6, 7 and 9 min (Fig. 3B,C) is unknown, but they were also detected only after Cd^{2+} exposure. Glutathione homologues such as γGluCysGlu with retention times between glutathione and PC-2 have been detected in maize (Meuwly et al., 1995).

The correlated redistribution of Cd and PCs, from the cytosol to the chloroplast after 8 days of Cd^{2+} exposure suggests transport of Cd-PC complexes. A similar reaction catalysed by an ATP binding cassette transporter (HMT1) has been characterized in the *Schizosaccharomyces pombe* vacuole (Ortiz et al. 1992). A bis(glutathionato) Cd^{2+} uptake reaction for vacuolar Cd sequestration has also been described in *Saccharomyces cerevisiae* (Li et al. 1997).

Although our work does not provide experimental support for a Cd-thiol peptide or Cd-glutathionate transport into the chloroplast, it does indeed demonstrate that isolated chloroplasts possess a transport system for the free Cd^{2+} ion. The partial temperature-dependence of the Cd^{2+} uptake suggested that, in addition to significant simple diffusion, there is also a protein-mediated Cd^{2+} transport in *Euglena* chloroplasts. No such kind of transport has been described in chloroplasts, although a Cd^{2+}/H^+ antiport activity in tonoplast vesicles from oat roots and in yeast vacuolar vesicles has been described (Salt and Wagner 1993, Ortiz et al. 1995). The existence of a free Cd^{2+} uptake system in *Euglena* chloroplast does not exclude the possibility of the presence of a Cd-complexed transporter. Another possibility is that Cd activates PC synthesis inside the chloroplast, although suborganellar distribution of a PC synthetase has not been reported.

The stoichiometry of thiol compounds (cysteine + glutathione + thiol-peptides)/Cd was 0.66 in purified chloroplasts. A similar stoichiometry was estimated for the cytosolic fraction. Since complete chelation of Cd^{2+} requires at least two thiol groups, our results indicated that the biosynthesis and/or uptake of these compounds was low and did not suffice to chelate the Cd^{2+} present in the chloroplasts. To completely account for resistance to this heavy metal, other chelating molecules such as dicarboxylic acids, inorganic phosphate, pyrophosphate and sulphide should also be involved in the inactivation of Cd^{2+} in *Euglena* chloroplasts.

Acknowledgements – Partial financial support from CONACyT-México (grants 26105-N and 25199-N) and DGAPA-UNAM (grant IN205697) is acknowledged.

References

Al-Lahham A, Rohde V, Heim P, Leuchter R, Veeck J, Wunderlich C, Wolf K, Zimmermann M (1999) Biosynthesis of phytochelatins in the fission yeast. Phytochelatin synthesis: a second role for the glutathione synthetase gene of *Schizosaccharomyces pombe*. Yeast 15: 385–396

Allen JF, Holmes NG (1986) Electron transport and redox titration. In: Hipkins MF, Baker NR (eds) Photosynthesis Energy Transduction: a Practical Approach, Chap. 5. IRL Press, Oxford, UK, pp. 103–141. ISBN 0-947946-63-2

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Plant Physiol 24: 1–15

Bariaud A, Bury M, Mestre JC (1985) Mechanism of Cd resistance in *Euglena gracilis*. Physiol Plant 63: 382–386

Bertaux O, Valencia R, Magnaval R (1989) The nucleus. In: Buetow DE (ed) The Biology of Euglena, Vol. IV, Chap. 2. Academic Press, New York, NY, pp 137–245. ISBN 0-12-139904-4

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Briand J, Calvayrac R (1980) Paramylon synthesis in heterotrophic and photoheterotrophic *Euglena* (Euglenophyceae). J Phycol 16: 234–239

Brunk UT, Dalen H, Roberg K (1997) Photooxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. Free Rad Biol Med 23: 616–626

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 18: 3325–3333

Cobbett CS (2000) Phytochelatins and their roles in heavy metal detoxification. Plant Physiol 123: 825–832

Coppellotti O (1989) Glutathione, cysteine and acid-soluble thiol levels in *Euglena gracilis* cells exposed to copper and cadmium. Comp Biochem Physiol 94C: 35–40

De Filippis LF, Hampp R, Ziegler H (1981) The effects of sublethal concentrations of zinc, cadmium and mercury on *Euglena*. II. Respiration, photosynthesis and photochemical activities. Arch Microbiol 128: 407–411

De Filippis LF, Pallaghy CK (1994) Heavy metals: sources and biological effects. In: Rai LC, Gaur JP, Soeder CJ (eds) Advances in Limnology Series: Algae and Water Pollution. Escheiweizerbartsche Press, Stuttgart, pp 31–77

Devars S, Hernández R, Moreno-Sánchez R (1998) Enhanced heavy metal tolerance in *Euglena gracilis* by preexposure to mercury or cadmium. Arch Environ Contam Toxicol 34: 128–135

Falchuk KH, Drishan A, Vallee BL (1975a) DNA distribution in the cell cycle of *Euglena gracilis*. Cytofluorometry of zinc deficient cells. Biochemistry 14: 3439–3444

Falchuk KH, Fawcett DW, Vallee BL (1975b) Competitive antagonism of cadmium and zinc in the morphology and cell division of *Euglena gracilis*. J Submicr Cytol 7: 139–152

Gekeler W, Grill E, Winnacker EL, Zenk MH (1988) Algae sequester heavy metals via phytochelatin complexes. Arch Microbiol 150: 197–202

Graham JM (1993) The identification of subcellular fractions from mammalian cells. In: Graham JM, Higgins JA (eds) Biomembrane Protocols I. Isolation and Analysis. Humana Press Inc., Totowa, New Jersey, pp 1–18. ISBN 0-89603-236-1

Grill E, Winnacker EL, Zenk MH (1991) Phytochelatins. Meth Enzymol 205: 333–341
Hell R, Bergmann L (1990) Glutamylcysteine synthetase in higher plants: Catalytic properties and subcellular localization. Planta 180: 603–612
Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele D, Rea P (1997) A new pathway for vacuolar sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis (glutathionato) cadmium. Proc Natl Acad Sci USA 94: 42–47
Lignell A, Roomans GM, Pedersen M (1982) Localization of cadmium in *Fucus vesiculosus* by X-ray microanalysis. Z Pflanzenphysiol Bd 105: 103–109
Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and *O*-acetylserine (thiol) lyase in spinach leaves. Plant Physiol 94: 1345–1352
Meuwly P, Thibault P, Schwan AL, Rauser WE (1995) Three families of thiol peptides are induced by cadmium in maize. Plant J 7: 391–400
Moreno-Sánchez R, Raya JC (1987) Preparation of coupled mitochondria from *Euglena* by sonication. Plant Sci 48: 151–157
Murphy JB, Kies MW (1960) Note on spectrophotometric determination of proteins in dilute solutions. Biochim Biophys Acta 45: 382–384
Nagel K, Adelmeier U, Voight J (1996) Subcellular distribution of cadmium in the unicellular green alga *Chlamydomonas reinhardtii*. J Plant Physiol 149: 86–90
Navarro L, Torres-Márquez ME, González-Moreno S, Devars S, Hernández R, Moreno-Sánchez R (1997) Comparison of physiological changes in *Euglena gracilis* during exposure to heavy metals of heterotrophic and autotrophic cells. Comp Biochem Physiol 116: 265–272
Nishimura M, Ito T, Chance B (1962) Studies on bacterial photophosphorylation. Biochim Biophys Acta 59: 177–182
Nussbaum S, Schmutz D, Brunold C (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays*. Plant Physiol 88: 1407–1410
Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow D (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J 11: 3491–5499
Ortiz DF, Ruscitti T, McKue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1: a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270: 4721–4728
Price CA, Cushman JC, Mendiola-Morgenthaler LR, Reardon EM (1987) Isolation of plastids in density gradients of Percoll and other silica solutions. Meth Enzymol 148: 157–179
Rauser WE (1990) Phytochelatins. Ann Rev Biochem 59: 61–86
Reed RH, Gadd GM (1989) Metal tolerance in eukaryotic and procaryotic algae.- In: Shaw AJ (ed) Heavy Metal Tolerance in Plants: Evolutionary Aspects, Chap. 8. CRC Press, Boca Raton Florida, pp. 105–118, ISBN 0-8493-6852-9
Saidha T, Na SQ, Li J, Schiff JA (1988) A sulphate metabolizing centre in *Euglena* mitochondria. Biochem J 253: 533–539
Salt DE, Wagner CJ (1993) Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd/H^+ antiport activity. J Biol Chem 268: 12297–12302
Schäfer HJ, Haag-Kerwer A, Rausch T (1998) CDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L. evidence for Cd-induction of a putative mitochondrial gamma-glutamylcysteine synthetase isoform. Plant Mol Biol 37: 87–97
Silverberg BA, Stokes PM, Ferstenberg LB (1976) Intracellular complexes in a copper-tolerant green alga. J Cell Biol 69: 210–214
Stallwitz E, Häder DP (1993) Motility and phototactic orientation of the flagellate *Euglena gracilis* impaired by heavy metal ions. J Photochem Photobiol 18: 67–74
Suzuki E, Tsuzuki M, Hiyachi S (1987) Photosynthetic characteristics of chloroplasts isolated from *Euglena gracilis* Z grown photoautothrophically. Plant Cell Physiol 28: 1377–1388
Trevors JT, Stratton GW, Gadd GM (1986) Cadmium transport, resistance and toxicity in bacteria, algae and fungi. Can J Microbiol 32: 447–464
Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium and lead. Ann Rev Biochem 41: 91–128
Zenk MH (1996) Heavy metal detoxification in higher plants - a review. Gene 179: 21–30

Edited by J. K. Schjoerring

Grill E, Winnacker EL, Zenk MH (1991) Phytochelatins. Meth Enzymol 205: 333–341
Heil R, Bergmann L (1990) Glutamylcysteine synthetase in higher plants: Catalytic properties and subcellular localization. Planta 180: 603–612
Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele D, Rea P (1997) A new pathway for vacuolar sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis (glutathionato) cadmium. Proc Natl Acad Sci USA 94: 42–47
Lignell A, Roomans GM, Pedersen M (1982) Localization of cadmium in *Fucus vesiculosus* by X-ray microanalysis. Z Pflanzenphysiol Bd 105: 103–109
Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and *O*-acetylserine (thiol) lyase in spinach leaves. Plant Physiol 94: 1345–1352
Meuwly P, Thibault P, Schwan AL, Rauser WE (1995) Three families of thiol peptides are induced by cadmium in maize. Plant J 7: 391–400
Moreno-Sánchez R, Raya JC (1987) Preparation of coupled mitochondria from *Euglena* by sonication. Plant Sci 48: 151–157
Murphy JB, Kies MW (1960) Note on spectrophotometric determination of proteins in dilute solutions. Biochim Biophys Acta 45: 382–384
Nagel K, Adelmeier U, Voight J (1996) Subcellular distribution of cadmium in the unicellular green alga *Chlamydomonas reinhardtii*. J Plant Physiol 149: 86–90
Navarro L, Torres-Márquez ME, González-Moreno S, Devars S, Hernández R, Moreno-Sánchez R (1997) Comparison of physiological changes in *Euglena gracilis* during exposure to heavy metals of heterotrophic and autotrophic cells. Comp Biochem Physiol 116: 265–272
Nishimura M, Ito T, Chance B (1962) Studies on bacterial photophosphorylation. Biochim Biophys Acta 59: 177–182
Nussbaum S, Schmutz D, Brunold C (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays*. Plant Physiol 88: 1407–1410
Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow D (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J 11: 3491–5499
Ortiz DF, Ruscitti T, McKue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1: a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270: 4721–4728
Price CA, Cushman JC, Mendiola-Morgenthaler LR, Reardon EM (1987) Isolation of plastids in density gradients of Percoll and other silica solutions. Meth Enzymol 148: 157–179
Rauser WE (1990) Phytochelatins. Ann Rev Biochem 59: 61–86
Reed RH, Gadd GM (1989) Metal tolerance in eukaryotic and procaryotic algae. In: Shaw AJ (ed) Heavy Metal Tolerance in Plants: Evolutionary Aspects, Chap. 8. CRC Press, Boca Raton Florida, pp. 105–118. ISBN 0-8493-6852-9
Saidha T, Na SQ, Li J, Schiff JA (1988) A sulphate metabolizing centre in *Euglena* mitochondria. Biochem J 253: 533–539
Salt DE, Wagner CJ (1993) Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd/H^+ antiport activity. J Biol Chem 268: 12297–12302
Schäfer HJ, Haag-Kerwer A, Rausch T (1998) CDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L. evidence for Cd-induction of a putative mitochondrial gamma-glutamylcysteine synthetase isoform. Plant Mol Biol 37: 87–97
Silverberg BA, Stokes PM, Ferstenberg LB (1976) Intracellular complexes in a copper-tolerant green alga. J Cell Biol 69: 210–214
Stallwitz E, Häder DP (1993) Motility and phototactic orientation of the flagellate *Euglena gracilis* impaired by heavy metal ions. J Photochem Photobiol 18: 67–74
Suzuki E, Tsuzuki M, Hiyachi S (1987) Photosynthetic characteristics of chloroplasts isolated from *Euglena gracilis* Z grown photoautothrophically. Plant Cell Physiol 28: 1377–1388
Trevors JT, Stratton GW, Gadd GM (1986) Cadmium transport, resistance and toxicity in bacteria, algae and fungi. Can J Microbiol 32: 447–464
Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium and lead. Ann Rev Biochem 41: 91–128
Zenk MH (1996) Heavy metal detoxification in higher plants – a review. Gene 179: 21–30

Edited by J. K. Schjoerring

Capitulo 5. Modelo de resistencia y acumulación de Cd^{2+} en el cloroplasto de *Euglena gracilis*.

Una vez determinado que el Cd^{2+} y las FQs se acumulaban en el cloroplasto (ver capitulo 4), el siguiente paso fue determinar el mecanismo bioquímico por el cual ambos compuestos se compartamentalizaban en este organelo. La figura 5.1 muestra el modelo que proponemos para explicar la acumulación de Cd^{2+} y FQs en el cloroplasto de *E. gracilis*.

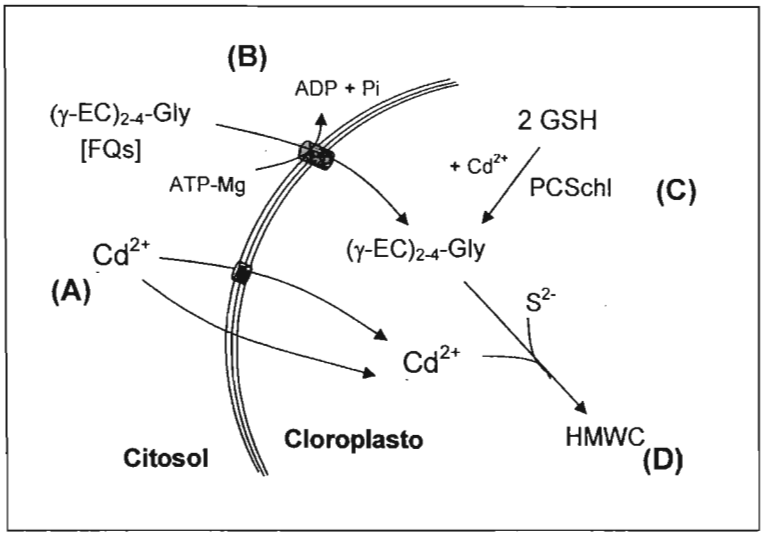


Figura 5.1 Modelo de resistencia a Cd^{2+} en *Euglena gracilis*.

Debido a que *E. gracilis* carece de una vacuola tipo planta y más del 60% del Cd^{2+} y FQs se encuentran en el cloroplasto (ver Capítulo 4; Mendoza-Cózatl et al., 2002), el Cd^{2+} podría entrar al cloroplasto como ión libre **(A)** y unido a FQs **(B)**, pero además, y debido a que el GSH está presente dentro del cloroplasto, propusimos que las FQs podrían sintetizarse también en el interior de este organelo **(C)**. Debido a que la inactivación completa del Cd^{2+} requiere de la formación de complejos de alto peso molecular formados por Cd^{2+} y tioles (HMWC), también nos planteamos como hipótesis que estos complejos podrían estar presentes en el cloroplasto de *Euglena* **(D)**. Como parte central del proyecto de Doctorado, nos propusimos entonces caracterizar los puntos **A**, **C** y **D**, de este modelo.

5.1 Hipótesis

El mecanismo de resistencia y acumulación de Cd^{2+} en *Euglena gracilis*, esta relacionado con su compartamentalización en el cloroplasto mediante la unión con diversos compuestos tioles (GSH, FQs y S^{2-}).

5.2 Objetivo general

Determinar el mecanismo de resistencia y acumulación de Cd^{2+} mediado por tioles en *Euglena gracilis*.

5.3 Objetivos particulares

1) Caracterizar cinéticamente el transporte de Cd^{2+} al cloroplasto de *Euglena gracilis* en forma de ión libre.

2) Determinar si la acumulación de Cd^{2+} en el cloroplasto de *Euglena gracilis* depende del contenido de tioles y sulfuro.

3) Determinar si las fitoquelatinas pueden sintetizarse en el interior del cloroplasto de *Euglena gracilis*.

4) Caracterizar fisicoquímicamente a los complejos de alto peso molecular formados por tioles, Cd^{2+} y sulfuro en *Euglena gracilis*.

Capitulo 6. Resultados

6.1 Caracterización cinética del transporte de Cd^{2+} en cloroplastos de *Euglena gracilis*

Como primer objetivo, nos centramos en caracterizar la entrada de Cd^{2+} al cloroplasto en forma de ion libre y en determinar si los tioles dentro del cloroplasto podrían estar relacionados con el mecanismo de resistencia y acumulación de Cd^{2+} en *E. gracilis*. Además de la entrada de Cd^{2+} al cloroplasto, también determinamos parte de la bioenergética del transporte. Finalmente, con base en las características bioquímicas del transporte, propusimos el tipo de transportador (a nivel de familia de transportadores) que podría estar mediando la entrada de Cd^{2+} al cloroplasto de *Euglena gracilis*.

p (59-68) Biochim. Biophys. Acta- Bioenergetics (2005) 1706: 88-9 and revision-acceptance letters

Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1706 (2005) 88–97
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*

David G. Mendoza-Cózatl*, Rafael Moreno-Sánchez

Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano #1, Col. Sección XVI, Tlalpan 14080, México, D.F., México

Received 7 April 2004; received in revised form 21 September 2004; accepted 22 September 2004
Available online 12 October 2004

Abstract

Euglena gracilis lacks a plant-like vacuole and, when grown in Cd^{2+}-containing medium, 60% of the accumulated Cd^{2+} is located inside the chloroplast. Hence, the biochemical mechanisms involved in Cd^{2+} accumulation in chloroplast were examined. Percoll-purified chloroplasts showed a temperature-sensitive uptake of the free $^{109}Cd^{2+}$ ion. Kinetics of the uptake initial rate was resolved in two components, one hyperbolic and saturable (V_{max} 11 nmol $^{109}Cd^{2+}$ min^{-1} mg protein $^{-1}$, K_m 13 μM) and the other, linear and non-saturable. $^{109}Cd^{2+}$ uptake was not affected by metabolic inhibitors or illumination. Zn^{2+} competitively inhibited $^{109}Cd^{2+}$ uptake (K_i 8.2 μM); internal Cd^{2+} slightly inhibited $^{109}Cd^{2+}$ uptake. Cadmium was partially and rapidly released from chloroplasts. These data suggested the involvement of a cation diffusion facilitator-like protein. Chloroplasts isolated from cells grown with 50 μM $CdCl_2$ (ZCd_{50} chloroplasts) showed a 1.6 times increase in the uptake V_{max}, whereas the K_m and the non-saturable component did not change. In addition, Cd^{2+} retention in chloroplasts correlated with the amount of internal sulfur compounds. ZCd_{50} chloroplasts, which contained 4.4 times more thiol-compounds and sulfide than control chloroplasts, retained six times more Cd^{2+}. The Cd^{2+} storage-inactivation mechanism was specific for Cd^{2+}, since Zn^{2+} and Fe^{3+} were not preferentially accumulated into chloroplasts.

Keywords: Heavy metal transport; Phytochelatin; Sulfide; Cd^{2+} compartmentation; Cd^{2+} inactivation

1. Introduction

Heavy metals such as Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} and Co^{2+} are essential micronutrients for cell metabolism but when present in excess, these, or trace amounts of nonessential metals such as Cd^{2+}, Hg^{2+}, Ag^{2+}, and Pb^{2+}, may become extremely toxic [1]. Metal tolerance and homeostasis in cells is achieved by controlling their transport processes and by their sequestration with specific metal-binding biomolecules such as phytochelatins (PCs) and metallothioneins [2,3].

In the plant cell, Cd^{2+} can enter through different pathways. In rice (*Oryza sativa*), maize (*Zea mays*) and root cells from soybean (*Glycine max*), Cd^{2+} uptake is a carrier-mediated process [4–6]. In barley (*Hordeum vulgare*), Cd^{2+} uptake is mainly a simple diffusion process [7]. In durum wheat roots (*Triticum turgidum*), Zn^{2+} and Cd^{2+} uptake is mediated by both a carrier and through simple diffusion [8,9].

At the protein level, five families have been implicated in heavy metal transport:

(1) CPx-type ATPases, a subgroup of the P-type ATPases with a Cys-Pro-X motif (CPx), which take up essential and nonessential metals such as Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} across the plasma membrane [1,10].
(2) Cation/H^+ antiporter family, which physiologically exchanges Na^+ or H^+ with Ca^{2+} or Cd^{2+} [11,12].
(3) Nramp, from *N*atural *r*esistance *a*ssociated *m*acrophage *p*rotein, first identified in mammalian cells and related to the susceptibility to infection by pathogens. These proteins are H^+-coupled transporters whose activity depends on the plasma membrane electrochemical H^+ gradient and have been involved in the transport of Cd^{2+}, Mn^{2+} and Cu^{2+} [11,12–15].

* Corresponding author. Tel.: +52 55 5573 2911; fax: +52 55 5573 0926.
E-mail address: cozatl@hotmail.com (D.G. Mendoza-Cózatl).

0005-2728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2004.09.010

(4) CDF (Cation *D*iffusion *F*acilitators) proteins related to Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} transport in bacteria, fungi, plants and animals [12,16]. Some of these proteins do not apparently require an energy source such as ATP or a H^+ motive force to function, since the metal concentration gradient between the two compartments is sufficient to drive the cation transport and, in consequence, this process may be reversible [17,18].

(5) ZIP, from ZRT/IRT-like *p*roteins. Plant transporters of Fe^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} with biochemical properties similar to those of CDF proteins, but with different topology and a highly conserved histidine motif for metal binding [19,20].

CDF and ZIP proteins have also been implicated in the intracellular transport of heavy metals. Zhf and hZnT-5, both members of the CDF family, facilitate the Zn^{2+} compartmentation into the endoplasmic reticulum and Golgi apparatus, in yeast and mammals, respectively [18,21]. In *S. cerevisiae*, ZRC1 and COT1, other CDF members, transport Zn^{2+}, Co^{2+} and probably Cd^{2+} into the yeast vacuole [1,17], whereas ZRT3, a ZIP family member, is involved in Zn^{2+} release from vacuole to cytosol [22]. In addition, the sequence of ZIP4, an *Arabidopsis* ZIP family member, contains a transit peptide for chloroplast, suggesting that this kind of proteins may also operate in the transport of heavy metals into this organelle [19,23]. However, the biochemical characterization of the CDF or ZIP family proteins involved in the transport of heavy metals to chloroplasts has not yet been carried out.

In plants and some yeast, it is well established that Cd^{2+} is accumulated into the vacuole. In plants, Cd^{2+} may be transported as free ion or as a phytochelatin-Cd (PC-Cd) complex [24,25]. In yeast, Cd^{2+} is also transported as a free ion and, depending on the species, it may also be transported as a PC-Cd or as a bis(glutathionato)-Cd complex (GS_2-Cd) [26,27]. In plants and yeast, the transport of the free Cd^{2+} ion requires a H^+ gradient across the vacuole membrane [24,26,27]; the transport of thiol-Cd complexes requires an ABC-type transporter specific for each kind of complex (PC-Cd or GS_2-Cd) [25–27]. Finally, for a complete Cd^{2+} inactivation and storage inside the vacuole, thiol(PCs, GSH or γ-EC)-Cd complexes, Cd^{2+} and inorganic sulfide (S^{2-}) form high molecular weight complexes (HMWC) around a CdS core [28,29]; cells unable to form these complexes become Cd^{2+}-sensitive [30,31].

Little is known about Cd^{2+} uptake and compartmentation in unicellular photosynthetic organisms. *Euglena gracilis* shows a high Cd^{2+} resistance associated with a large Cd^{2+} accumulation capacity; the $CdCl_2$ concentration to reach 50% growth inhibition is 100 μM whereas cells cultured in the presence of 200 μM $CdCl_2$ accumulate 249 nmol Cd^{2+}/10^7 cells (2.79 μg Cd^{2+}/mg protein) [32]. *E. gracilis* lacks a plant-like vacuole and thus more than 60% of the accumulated Cd^{2+} is located inside the chloroplast [32]. PCs are also present inside the *E. gracilis* chloroplast [32], suggesting that this organelle is involved in the Cd^{2+} resistance mechanism. The understanding of the biochemical mechanisms for heavy metal resistance and accumulation in organisms such as *E. gracilis* is of interest for bioremediation purposes. Therefore, in the present work the Cd^{2+} uptake and the Cd^{2+} storage mechanism of Percoll-purified chloroplasts isolated from photosynthetic *E. gracilis* were evaluated.

2. Materials and methods

2.1. Chemicals

EGTA, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (DCMU) were purchased from Sigma. HEPES was from Boehringer, Percoll from Pharmacia, sorbitol from Research Organics and $^{109}CdCl_2$ was from NEN (specific activity 5550–7400 Bq μmol^{-1}).

2.2. Cell culture and growth conditions

Axenic cultures of *E. gracilis* Klebs (strain Z) were grown under cycles of 12 h of fluorescent white light (70 μmol quanta m^{-2} s^{-1}) and 12-h dark at 25±2 °C with no agitation. The acidic organotrophic medium with 33 mM glutamate and 17 mM malate as carbon source, initial pH 3.5, was used [33]; the medium pH remained unchanged for the first 72 h of culture, reaching a value of 5.0 (n=2) after 8 days. The culture medium also contained the following salts: 3.2 mM $(NH_4)_2HPO_4$, 5.6 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCO_3$, 473 μM $MnSO_4 \cdot 4H_2O$, 306 μM $ZnSO_4 \cdot 7H_2O$, 90 μM $Na_2MoO_4 \cdot 2H_2O$, 18.4 μM $FeCl_3 \cdot 6H_2O$, 6.7 μM $CoCl_2 \cdot 6H_2O$, 6.2 μM $CuSO_4 \cdot 5H_2O$, 0, 1.7 μM NaI.

$CdCl_2$ was added to the culture medium before the cellular inoculum. At a pH of 3.5 and in the presence of 33 mM glutamate, 17 mM malate, 8.86 mM $H_2PO_4^-$, 2 mM Mg^{2+} and 2 mM Ca^{2+}, the chemical activity of 50 and 100 μM added Cd^{2+} was 40.2 and 80.1 μM free Cd^{2+}, respectively. These latter values were estimated by using the software CHELATOR [34] for multiple ligand equilibrium reaction systems and the stability constants compiled by Sillen and Martell [35]. Free Cd^{2+} concentration at 50 μM of added $CdCl_2$ was also experimentally determined to be 41.7 μM free Cd^{2+} in a medium that contained 33 mM glutamate, 17 mM malate, 8.86 mM $H_2PO_4^-$, pH 3.5, and 1 μM of the fluorescence dye Mag-Fura-2 (Molecular Probes). The Kd_{Cd} of the Cd^{2+}-dye complex was 1.8±0.2 μM (also experimentally determined at pH 3.5). The probe was stable (>96%) at the pH used during the measurements. Excitations wavelengths were 323 and 358 nm and emission was

collected at 483 nm; data were processed as described elsewhere [36].

2.3. Isolation of chloroplasts

Intact Percoll-purified chloroplasts were obtained as described previously [32] with some modifications. After separation of the intact chloroplasts from the 40–80% Percoll interface, chloroplasts were washed once with 10 volumes of a medium that contained 0.33 M sorbitol, 10 mM HEPES, pH 7.3 and centrifuged at 2000×*g* for 5 min. Then, chloroplasts were resuspended in a minimal volume of the same buffer, stored in ice, and used within the next 2 h for the uptake experiments. Chloroplasts showed membrane integrity higher than 95%, as determined by the Hill reaction.

2.4. Cd^{2+} uptake and release experiments

$^{109}Cd^{2+}$ uptake was measured as described previously [32] with some modifications. Uptake was assayed at 25 °C in a medium that contained 0.33 M sorbitol, 10 mM HEPES, pH 7.3. To ensure initial uptake rate conditions, 0.1 mg of chloroplast protein (in 0.5 ml) was withdrawn after 20-s incubation with $^{109}CdCl_2$ and filtered through nitrocellulose filters (0.45-μm pore diameter, Sartorius, Germany) previously rinsed with 10 mM $CdCl_2$. Under these conditions, a linear rate was attained from 10 to 30 s for $CdCl_2$ concentrations of 50–200 μM. Then, filters were washed twice with 10 ml of ice-cold 10 mM $CdCl_2$. Radioactivity in the filters was measured in a liquid scintillation counter. Filters washed with either 120 mM KCl, 1 mM DTT or 10 mM EGTA yielded similar results. Kinetic data were fitted to Eq. (1) (Michaelis–Menten plus a linear component) using the Microcal Origin 5.0 (Microsoft) software.

$$v = \left(\frac{V_m[Cd^{2+}]}{K_m + [Cd^{2+}]}\right) + m[Cd^{2+}] \quad (1)$$

For $^{109}Cd^{2+}$ loading, 0.5 ml of a chloroplast suspension of 4 mg protein/ml was incubated for 10 min at 25 °C, or the stated time, with 100 μM $^{109}CdCl_2$. For the release experiments, aliquots of $^{109}CdCl_2$-loaded chloroplasts were transferred to a release medium (0.33 M sorbitol, 10 mM HEPES with or without a competing metal or 10 mM EGTA, pH 7.3) to a final concentration of 0.2 mg protein/ml; after the indicated times, 0.1 mg of chloroplast protein (in 0.5 ml) was withdrawn, filtered and washed for radioactivity counting.

2.5. Cadmium, zinc and iron content

Content of heavy metals in acid-digested cells (10^7–10^8 cells) and chloroplasts (5–10 mg protein) was determined by atomic absorption spectrophotometry in a SpectrAA 640 (Varian Australia Pty) spectrophotometer.

2.6. Content of acid soluble thiol-compounds and sulfide

Cellular and chloroplast contents of cysteine (Cys), γ-glutamylcysteine (γ-EC), reduced glutathione (GSH) and phytochelatins (PCs) were determined by reverse phase-HPLC (RP-HPLC) coupled to post-column derivatization with DTNB (Ellman's reagent) as described in [37], with some modifications. To separate γ-EC from GSH, an initial elution step with 0.1% (v/v) trifluoroacetic acid in water for 10 min was carried out, which was followed by a linear gradient of 0–20% acetonitrile. Acid-labile sulfide was determined as described in Ref. [38].

3. Results

3.1. Cd^{2+} uptake

We previously reported that Cd^{2+} enters the chloroplast as a free ion [32]; therefore, in this work we characterized the mechanism by which Cd^{2+} is transported and retained inside the chloroplast. Initial rates of $^{109}Cd^{2+}$ uptake in Percoll-purified chloroplasts of *E. gracilis* showed a biphasic, non-saturating pattern (Fig. 1A). Double-reciprocal plots yielded downward, hyperbolic curves (not shown), suggesting that at least two components were involved in the uptake process. By nonlinear regression fitting to Eq. (1), Cd^{2+} uptake was dissected into two components, a saturable (hyperbolic) and a non-saturable one (linear). The saturable component showed a V_{max} of 11.2±3 (4) nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$ [mean±S.D. (*n*)] and a K_m of 13.4±5 (4) μM. The rate of the non-saturable component was 0.13±0.01 (4) nmol $^{109}Cd^{2+}$ min^{-1} mg $protein^{-1}$ μM^{-1}, representing the increase in the Cd^{2+} uptake rate related to the increase in the Cd^{2+} concentration in the assay. Eq. (1) was the best fit to the experimental data of Fig. 1A over the Hill, double Michaelis–Menten or double Hill equations (not shown).

To determine the nature of the driving force involved in the Cd^{2+} uptake, different inhibitors were tested. At 100 μM $^{109}CdCl_2$, the uptake initial rate was 21.4±4.6 (4) nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$; this rate was not affected by the addition of 100 μM CCCP [19±4.2 (4) nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$] or 50 μM DCMU [19.4±3 (4) nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$]. These results suggested that Cd^{2+} uptake did not depend on an electrochemical H^+ gradient across the chloroplast envelope or on the photosynthetic electron transport activity. Mersalyl (50 μM), ruthenium red (100 μM), pre-illumination of chloroplasts or pre-incubation in the dark did not affect $^{109}Cd^{2+}$ uptake (data not shown).

To further assess whether an ion electrochemical gradient was involved, the K^+/H^+ ionophore nigericin was also assayed. Nigericin showed no effect on $^{109}Cd^{2+}$ uptake in the absence of added K^+, but 50% inhibition by nigericin was attained in the presence of 150 mM KCl [21.4 nmol

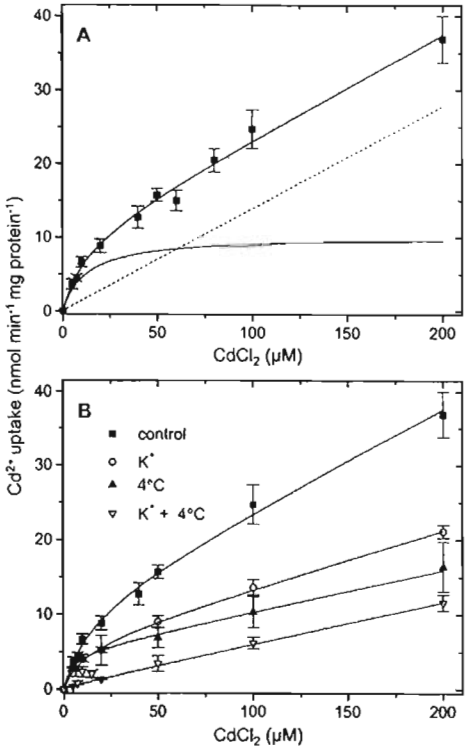


Fig. 1. (A) Kinetics of $^{109}Cd^{2+}$ uptake in Percoll-purified chloroplasts from *E. gracilis*. $^{109}Cd^{2+}$ uptake was measured by filtration as described under Materials and methods. The data shown represent mean±S.D. of four to five different preparations. Kinetic parameters were calculated by fitting the experimental data to Eq. (1) (thick solid line). Hyperbolic (thin solid line) and linear (dotted line) components derived from the nonlinear regression analysis are also shown. (B) Effect of temperature and K^+ on the $^{109}Cd^{2+}$ uptake kinetics. Chloroplasts were pre-incubated for 5 min in a medium containing 0.33 M sorbitol, 10 mM HEPES (pH 7.3), at 25 (■) or 4 °C (▲), with 50 mM KCl at 25 °C (○), or with 50 mM KCl at 4 °C (▽). Uptake was initiated by the addition of $^{109}CdCl_2$ at the indicated concentrations. After 20 s, an aliquot of chloroplasts was withdrawn, filtered, washed and the radioactivity in the filters was measured in a liquid scintillation counter.

$^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$ vs. 11.1±3 (4) nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$]. Because K^+ is a physiological cation present in cytosol and chloroplast, the K^+ effect on the Cd^{2+} uptake kinetics was examined (Fig. 1B). K^+ inhibited both components, the saturable and the non-saturable, by decreasing their V_{max} and slope values, respectively, but the K_m value remained unchanged (Table 1A).

The effect of temperature was also tested (Fig. 1B). Temperature affected both components in a greater proportion than K^+, and all three kinetic parameters (V_{max}, K_m, slope) were affected (Table 1A). Temperature and K^+ showed an additive effect (Fig. 1B), eliminating almost completely the saturable component (Table 1A). Indeed, in the presence of 50 mM KCl and at 4 °C, the kinetic pattern fitted to a linear equation starting at the origin.

It has been demonstrated that exposure to heavy metals affects the expression of some heavy metal transporters [19,20]. Therefore, $^{109}Cd^{2+}$ uptake in chloroplasts isolated from cells growing for eight days with 50 or 100 μM $CdCl_2$ (ZCd_{50} and ZCd_{100} chloroplasts) was also studied. In these chloroplasts, a significant 57–61.6% increase in V_{max} was found (Table 1B). The K_m of the saturable component and the rate (slope) of the non-saturable component remained unchanged. Similar chlorophyll to protein ratios were found in chloroplasts from control and Cd^{2+}-grown cells, discarding an unspecific and generalized increase in the protein content as related to the V_{max} increase (data not shown).

3.2. Effect of divalent cations on the Cd^{2+} uptake

To enter cells and organelles, nonessential heavy metals use the essential heavy metal transporters. Hence, it may be expected that different divalent cations may inhibit the Cd^{2+} transport. Depending on the effective inhibiting cations, it would be possible to elucidate which transporter might be mediating the Cd^{2+} uptake in *Euglena* chloroplast. Zn^{2+} significantly inhibited $^{109}Cd^{2+}$ uptake whereas Mn^{2+} and Mg^{2+} showed no effect; Ba^{2+} and Ca^{2+} only showed an inhibitory effect in control chloroplasts (Fig. 2A).

Zn^{2+} inhibited the $^{109}Cd^{2+}$ uptake in a concentration-dependent manner, but above 200 μM no further effect was attained (Fig. 2B). Dixon plots (1/*v* vs. Zn^{2+}) yielded downward curves (not shown), suggesting either partial inhibition or that two components were involved in Cd^{2+} uptake but only one of them was inhibited by Zn^{2+}. The last possibility seemed more feasible since kinetic analysis showed that two components were mediating the Cd^{2+}

Table 1
Kinetic parameters of $^{109}Cd^{2+}$ uptake in chloroplasts of *E. gracilis*

	V_{max} (nmol Cd^{2+} min^{-1} mg protein^{-1})	K_m (μM)	Slope (nmol Cd^{2+} min^{-1} mg protein^{-1} μM^{-1})
(A) Effect of K^+ and temperature on $^{109}Cd^{2+}$ uptake in control chloroplasts			
Control	11.3±2	13.5±3	0.13±0.01
K^+	6.2±1	11.4±3	0.076±0.006
4 °C	4.9±0.4	4.4±0.7	0.05±0.004
K^++4 °C	ND	ND	0.05±0.01
(B) Kinetic parameters of chloroplasts isolated from control and Cd^{2+}-exposed cells			
Control	11.2±3	13.4±5	0.15±0.03
ZCd_{50} Chloroplasts	18.1±2*	12.6±1.4	0.12±0.04
ZCd_{100} Chloroplasts	17.6	19	0.12

(A) Kinetic parameters were calculated from the uptake experiments shown in Fig. 1 by nonlinear regression using Eq. (1). (B) Initial rates of $^{109}Cd^{2+}$ uptake in chloroplasts, isolated from cells grown for eight days without (control) and with 50 or 100 μM $CdCl_2$. Results are mean±S.D. of 3–4 different preparations for control and ZCd_{50} chloroplasts and 2 preparations for ZCd_{100} chloroplasts. *P≤0.05 versus control chloroplasts. ND, not detected.

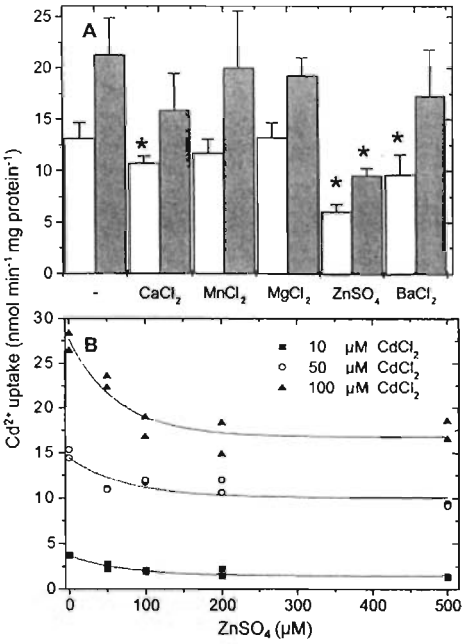


Fig. 2. (A) Effect of divalent cations on $^{109}Cd^{2+}$ uptake. Initial $^{109}Cd^{2+}$ uptake rates in control (open bars; B) and ZCd_{50} (filled bars) chloroplasts were assayed at 50 μM $^{109}CdCl_2$ and 200 μM of the indicated metals. Results are mean±S.D. of four to five different preparations and significant differences (*$P<0.05$) are indicated. (B) Concentration-dependent inhibition of $^{109}Cd^{2+}$ uptake by Zn^{2+}. The asymptotic values used to estimate the Cd^{2+} uptake rate that was resistant to Zn^{2+} were 1.2 (for 10 μM Cd^{2+}), 9.1 (for 50 μM) and 14.8 (for 100 μM) nmol Cd^{2+} min^{-1} mg $protein^{-1}$. Similar values may be obtained by using the slope of the linear component of Fig. 1A at the corresponding $CdCl_2$ concentrations.

uptake into *Euglena* chloroplast (Fig. 1A). Therefore, assuming that Zn^{2+} did not inhibit the non-saturable component and hence it did inhibit the saturable component, the data of Fig. 2B were corrected by subtracting the corresponding rates of $^{109}Cd^{2+}$ uptake resistant to Zn^{2+}. Simple competitive inhibition showed the best fit to these corrected rates over mixed and noncompetitive inhibition (not shown), with a K_i value of 8.2±2 μM. These results suggested that a Zn^{2+} transporter in the chloroplast envelope might be mediating the Cd^{2+} uptake in *Euglena* chloroplasts.

3.3. Cd^{2+} release

The time-dependent release of $^{109}Cd^{2+}$ from control chloroplasts was also analyzed (Fig. 3A). It should be noted that the amount of accumulated $^{109}Cd^{2+}$ in these experiments was lower than in the previous kinetics assays. This was probably due to the high protein concentration (20-fold more) used in the $^{109}Cd^{2+}$-loading, increasing the unspecific binding and reducing the free Cd^{2+} concentration. Indeed, a significant amount of $^{109}Cd^{2+}$ was bound to the external chloroplast surface (4.6 nmol $^{109}Cd^{2+}$/mg protein; n=2), which was efficiently removed by washing the chloroplasts retained in the filters with ice-cold $CdCl_2$ (see Materials and methods). No differences were found between washing the chloroplasts with an excess of $CdCl_2$ (10 mM) or with KCl (120 mM) or EGTA (10 mM). Therefore, most of the $^{109}Cd^{2+}$ retained by the chloroplasts was very likely inside the organelle.

Cadmium release was too fast to allow an accurate estimation of the release initial rates (Fig. 3A). It was preferred to refer to it as cadmium since the nature of the released component (as free ion or as a complex with thiol-compounds) was not determined. However, it was interesting to note that the cadmium release was only partial. This is

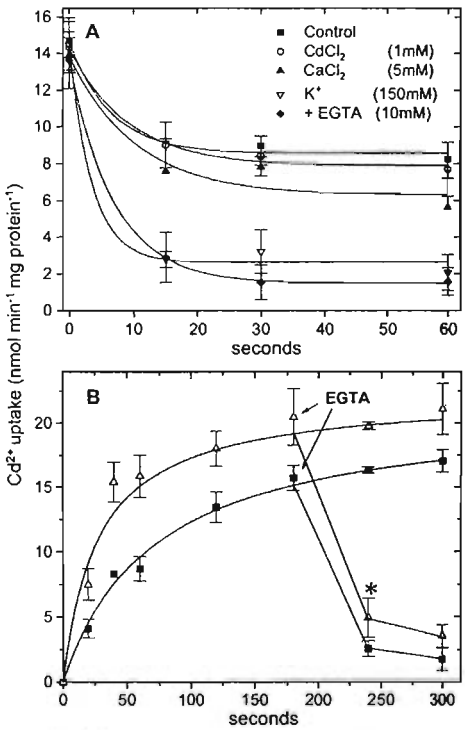


Fig. 3. $^{109}Cd^{2+}$ release in *E. gracilis* chloroplasts. (A) Chloroplasts were loaded with $^{109}CdCl_2$ as described under Materials and methods. After 10 min, the suspension was diluted 20 times with fresh medium (■) or medium containing (○) 1 mM $CdCl_2$, (▲) 5 mM $CaCl_2$, (▽) 159 mM KCl or (◆) 10 mM EGTA. At the indicated times, an aliquot was withdrawn, filtered for radioactivity measurement and washed twice with 8 ml of ice-cold 10 mM $CdCl_2$. (B) $^{109}Cd^{2+}$ uptake in control (■) and ZCd_{50} chloroplasts (△) was initiated by the addition of 50 μM $^{109}CdCl_2$ after 3 min. 10 mM EGTA was added. Aliquots of chloroplasts were withdrawn at different times, filtered and washed for radioactivity measurements. Results are mean±S.D. of four to five experiments and significant differences (*$P<0.05$) are indicated.

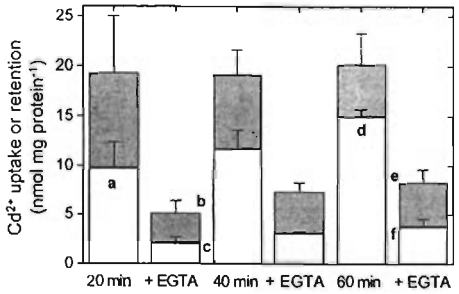


Fig. 4. $^{109}Cd^{2+}$ retention by *E. gracilis* chloroplasts. Control (open bars) and ZCd_{50} chloroplasts (filled bars) were loaded with $^{109}CdCl_2$ as described under Materials and methods. At the indicated times, an aliquot equivalent to 0.1 mg protein was withdrawn to determine the Cd^{2+} accumulation. In parallel, another aliquot was transferred to a medium containing 0.33 M sorbitol, 10 mM HEPES, 10 mM EGTA (pH 7.3) to a final concentration of 0.2 mg protein/ml; after 60 s, 0.5 ml (0.1 mg protein) was filtered to evaluate the amount of $^{109}Cd^{2+}$ retained. Results are mean±S.D. of four to five experiments. Significant differences ($P<0.05$) between a vs. d; b vs. c; e vs. f; b vs. e; and e vs. f were found.

expected if a protein acts as a diffusion facilitator and equilibrates the Cd^{2+} concentration between two compartments. To test this last hypothesis, the following experiments were carried out.

First, $^{109}Cd^{2+}$ uptake in Cd^{2+}-loaded chloroplasts was assayed. Control chloroplasts, loaded with 0.1 mM nonradioactive Cd^{2+} for 60 min at 25 °C, showed a decreased $^{109}Cd^{2+}$ uptake initial rate [15.6±0.3 (n=3) versus 13.3±0.8 (n=3) nmol $^{109}Cd^{2+}$ min^{-1} mg $protein^{-1}$ at 50 μM $^{109}CdCl_2$; $P<0.01$, Student's t-test for nonpaired samples]. Second, the presence of added nonradioactive Cd^{2+}, or Ca^{2+}, in the release medium did not increase the cadmium release (Fig. 3A). In contrast, chloroplasts diluted in a medium containing either EGTA, to diminish the external $^{109}Cd^{2+}$ concentration, or KCl, to block $^{109}Cd^{2+}$ re-entry, showed an increased release of cadmium, although part of the loaded $^{109}Cd^{2+}$ still remained inside the chloroplasts (Fig. 3A and B). It was also noted in the release experiments with EGTA that ZCd_{50} chloroplasts retained significantly more $^{109}Cd^{2+}$ than control chloroplasts (Fig. 3B). These results suggested that Cd^{2+} transport was reversible, but another process, such as equilibration between Cd^{2+}-binding molecules and free Cd^{2+}, might also be taking place for long-term Cd^{2+} accumulation inside the chloroplast.

Therefore, to test this last proposal, both types of chloroplasts were incubated with $^{109}Cd^{2+}$ for longer periods, to evaluate their Cd^{2+}-retention capacity (Fig. 4). After 20 min, $^{109}Cd^{2+}$ accumulation increased only in control chloroplasts, but $^{109}Cd^{2+}$ content was higher in ZCd_{50} chloroplasts. From 20 to 60 min of incubation, $^{109}Cd^{2+}$ retention was a time-linear function in both types of chloroplasts, and was higher in ZCd_{50} chloroplasts.

3.4. Acid-soluble organic thiol-compounds and inorganic sulfide in chloroplasts

Among the different functional groups of biomolecules (-NH_2, -COOH, -OH, -OPO_3^{2-} and -SH), the thiol group has the highest affinity for Cd^{2+} [35]. On the other hand, Cd^{2+} exposure (0.2 mM) induces PCs synthesis and an increase in the Cys and GSH content in *E. gracilis* chloroplasts [32]. It follows that Cys, GSH and PCs may very likely be the Cd^{2+}-binding compounds inside the chloroplast that mostly account for Cd^{2+} retention. To test this hypothesis, soluble acid thiol-compounds were determined in extracts from both types of chloroplasts. As expected, ZCd_{50} chloroplasts exhibited a higher content of acid soluble thiol-compounds than control chloroplasts (Table 2). In control chloroplasts, Cys was the most abundant organic thiol-compound and PCs levels were negligible. In ZCd_{50} chloroplasts, a significantly higher amount of PCs was found, but GSH and γ-EC were the most abundant thiol-compounds representing 66% of the total organic thiol content.

The higher amount of $^{109}Cd^{2+}$ retained after 60 min of incubation with 0.1 mM $^{109}CdCl_2$ correlated well with the higher amount of organic thiols and inorganic sulfide present in ZCd_{50} chloroplasts (Table 2). Although such relationship was not strictly proportional, it should be noted that, before the $^{109}Cd^{2+}$ incubation, ZCd_{50} chloroplasts already contained a considerable amount of internal Cd^{2+} (9.3±0.9 nmol Cd^{2+}/mg protein), brought about from growing cells in the presence of $CdCl_2$. Therefore, the total Cd^{2+} retention should be considered as the sum of the $^{109}Cd^{2+}$ retained from the 60-min incubation (8.25 nmol Cd^{2+}/mg protein) plus the Cd^{2+} brought about from the cell culture. Following these considerations, the Cd^{2+}/-SH ratio was 3.7 and 3.9 for control and ZCd_{50} chloroplasts, respectively. Since full inactivation of Cd^{2+} requires a Cd^{2+}/-SH ratio of 0.5 or lower, it seemed that other molecules such as inorganic sulfide and organic acids

Table 2
Thiol-containing molecules, sulfide and cadmium content in control and ZCd_{50} chloroplasts

Chloroplasts	Cys	GSH	γ-EC	PC_2	PC_3	PC_4	Total thiols	Sulfide	Cd^{2+}
	nmol/mg protein								
Control	0.44±0.1	0.33±0.2	0.3±0.1	0.028±0.005	ND	ND	1.01±0.4	0.95±0.4	0
ZCd_{50}	0.5±0.06	1.5±0.3	1.3±0.3	0.5?±0.1	0.44±0.1	0.18±0.1	4.5±0.9	4.2±1.7	9.3±0.9

Thiol-compounds in freshly prepared Percoll-purified chloroplasts were quantified by RP-HPLC. PCs are reported on thiol-content basis. Cadmium content was determined by atomic absorption spectrophotometry and sulfide by the methylene blue method [38]. Results are mean±S.D. of four to five experiments. ND, not detected.

Table 3
Cadmium, iron and zinc content in chloroplasts from control and Cd^{2+} exposed cells

	nmol metal/10^7 cells	nmol metal/μg Chl	Metal content in chloroplast
Cadmium			
ZCd_{50} Cells	70±14 (4)	–	–
ZCd_{50} Chloroplasts	–	0.92±0.1 (3)	62.7%
Iron			
Control Cells	37±4.8 (3)	–	–
Control Chloroplast	–	0.38±0.1 (3)	45%
ZCd_{50} Cells	57±17 (3)	–	–
ZCd_{50} Chloroplasts	–	0.34±0.1 (3)	28.5%
Zinc			
Control Cells	157±50 (3)	–	–
Control Chloroplast	–	0.93±0.3 (3)	26%
ZCd_{50} Cells	202±55 (3)	–	–
ZCd_{50} Chloroplasts	–	0.67±0.2 (3)	15.8%

The contents of cadmium, zinc and iron in chloroplasts isolated from cells grown for 8 days without or with 50 μM $CdCl_2$ were estimated by using the amount of metal per cell unit (10^7 cells), and the metal/μg Chl ratio in Percoll-purified chloroplasts. The chlorophyll content was 43.8 and 47.7 μg Chl/10^7 cells, for control and ZCd_{50} cells, respectively. Results are mean±S.D. of three to four different preparations.

should also be involved in achieving an efficient Cd^{2+} inactivation inside the chloroplast.

Cells grown with 50 μM $CdCl_2$ showed a sevenfold higher content of sulfide than control cells [34.9 versus 5.1 nmol $S^{2-}/10^7$ cells (n=2)]. ZCd_{50} chloroplasts also contained four times more sulfide than control chloroplasts (Table 2). Therefore, sulfide could contribute, together with PCs, GSH, Cys and γ-EC, to Cd^{2+} inactivation inside the chloroplasts.

3.5. Zn^{2+} and Fe^{3+} content in chloroplasts

As Cd^{2+} was mainly located inside the *Euglena* chloroplast [32], it might be expected that other heavy metals would also be preferentially located in this organelle. Although significant amounts of Zn^{2+} and Fe^{3+} were found in both types of cells and chloroplasts (Table 3), intracellular Zn^{2+} and Fe^{3+} were not predominantly compartmentated into chloroplasts (Table 3). In contrast, Cd^{2+} was mainly located in chloroplasts (62.8%).

4. Discussion

Kinetics of the initial uptake rate of free Cd^{2+} into *E. gracilis* chloroplasts was resolved in two components, one saturable and another non-saturable. The saturable component was highly sensitive to temperature, K^+ and Zn^{2+}, suggesting that it may be a protein of the chloroplast envelope. The nature of the non-saturable component was probably related either to passive diffusion through the organelle membranes or to unspecific transport through ion channels [39]. Ca^{2+} channels have been associated with the Cd^{2+} uptake across the plasma membrane in a pituitary cell line [39]; similar Ca^{2+} channels have been found in the inner envelope of plant chloroplasts [40].

In previous work [32], analysis of Cd^{2+} uptake in ZCd_{50} chloroplasts after incubation for 5–30 min revealed only one saturable component with a K_m value of 57 μM and V_{max} of 3.7 nmol $^{109}Cd^{2+}$ min^{-1} (mg protein)$^{-1}$. In the present work, by adjusting the protein concentration and the time of incubation, the *initial* uptake rate was analyzed, thus revealing that the affinity and V_{max} of the saturable component were in fact 4.3 and 3 times higher, respectively (cf. Table 1). According to the results of the present work, the previous kinetic analysis [32] also involved the Cd^{2+} retention processes of thiol-compounds and HMWC formation.

Taking into consideration the amount of Cd^{2+} available in uncontaminated and contaminated soils, cytosolic free Cd^{2+} concentrations in plants have been estimated of the order of 0.005 to 2 μM [24]. The K_m value obtained in this work (cf. Table 1), although higher than the estimated free Cd^{2+} in cytosol, was in agreement with other K_m values reported for Cd^{2+} carrier proteins (0.020 to 33 μM) [9,24,41]. By using Eq. (1), it may be predicted that at 2 μM free Cd^{2+}, the uptake rate in *E. gracilis* chloroplasts would be 1.76 nmol Cd^{2+} min^{-1} (mg protein)$^{-1}$, with 83% of the uptake carried out by the saturable component and only 17% by simple diffusion. Therefore, Cd^{2+} accumulation in the chloroplast of *E. gracilis* would be mainly a protein-mediated process at low free Cd^{2+} (<10 μM) concentrations.

The lack of effect of either pre-illumination, darkness or several metabolic inhibitors such as CCCP, DCMU, mersalyl or nigericin on $^{109}Cd^{2+}$ uptake indicated that Cd^{2+} transport did not require a H^+ gradient across the chloroplast envelope or the photosynthetic electron transport chain activity. Inhibition of $^{109}Cd^{2+}$ uptake by high, although physiological concentrations of K^+ indicated an unspecific effect, probably related to a saturation of negatively charged sites in the chloroplast envelope.

$^{109}Cd^{2+}$-loaded chloroplasts showed a rapid cadmium release (Fig. 3A and B), which was not stimulated in chloroplasts diluted in a medium containing a high concentration of a competing divalent ion such as Ca^{2+} or Cd^{2+} itself. This last observation supported the notion that the main part of cadmium was inside the chloroplast and not loosely bound to the external chloroplast surface. On the other hand, EGTA, by decreasing the external $^{109}Cd^{2+}$ concentration, and KCl, by inhibiting $^{109}Cd^{2+}$ uptake and by saturating the negatively charged sites in the chloroplast envelope, promoted an increased cadmium release. Thus, EGTA or KCl apparently altered the Cd^{2+} electrochemical equilibrium across the chloroplast envelope. The reversibility of the process suggested that the Cd^{2+} gradient across the chloroplast envelope was apparently sufficient to drive net Cd^{2+} transport. This explanation was further supported

by the small, but significant inhibition attained by internal Cd^{2+} on $^{109}Cd^{2+}$ uptake. Therefore, this Cd^{2+} transport process should be considered as facilitated diffusion.

Proteins that catalyze a facilitated diffusion of heavy metals such as Zn^{2+} and Cd^{2+} belong to the CDF and ZIP protein families. They have been found in all kingdoms [12], including protists [23], but only few of them have been characterized at the biochemical level. They have been preferentially located in the plasma membrane and, due to the ion (K^+, H^+) electrochemical gradient across the cell membrane (negative inside), the heavy metal uptake through these proteins is not generally considered as facilitated diffusion [17]. However, proteins from both families have also been found in organelles in which facilitated diffusion of heavy metals is accepted [18,21]. Moreover, analysis of the *Arabidopsis* ZIP4 amino acid sequence shows a chloroplast transit peptide [19,23], suggesting that it could mediate heavy metal transport into that organelle.

Protein-mediated transport of heavy metals that is not dependent on a H^+ gradient has also been reported in liver and prostate mitochondria [42], pancreatic β-cells [18] and in proteoliposomes containing a CDF protein from *A. thaliana* [17]. CDF proteins are mostly considered as cation efflux proteins whereas ZIP proteins are involved in the heavy metal uptake process [12,17,43]. The strong and competitive inhibition of $^{109}Cd^{2+}$ uptake in *E. gracilis* chloroplasts by Zn^{2+} suggested that a Zn^{2+} transporter, perhaps belonging to the CDF or ZIP protein families, might mediate $^{109}Cd^{2+}$ transport.

The genetic expression of several Zn^{2+} transporters is regulated by Zn^{2+} availability [19,20]. Cd^{2+} exposure may result in an altered Zn^{2+} sensing ability. Interestingly, chloroplasts isolated from cells grown with $CdCl_2$ showed a slight, but significantly higher V_{max} for Cd^{2+} uptake, implying an increase in the transporter content.

In plants and some yeast, PCs, Cd^{2+} and sulfide form HMWC which are essential for Cd^{2+} accumulation and inactivation in vacuoles [29–31,44,45]. These complexes have a Cd^{2+}/-SH molar ratio of 1, whereas the Cd^{2+}/S^{2-} ratio is more variable, depending on the organism, ranging from 0.5 to 5.5 [29,31,44].

As shown in this (cf. Table 2) and in previous work [32], PCs and sulfide are present in *E. gracilis* chloroplasts. These molecules may be mediating the Cd^{2+} storage-inactivation process inside the chloroplast since the Cd^{2+}/-SH and Cd^{2+}/S^{2} ratios (2 and 2.2, respectively) were similar to those found in plants [29,44]. Furthermore, chloroplasts isolated from cells grown with $CdCl_2$ showed enhanced levels of organic thiol-compounds and inorganic sulfide as well as a higher ability to accumulate Cd^{2+}. Cys, γ-EC and GSH contributed to the Cd^{2+} inactivation process since they accounted for 66% of the acid-soluble thiol-compounds in the stroma (Table 2). HMWC of plants and yeast do not contain Cys or GSH; in contrast, HMWC isolated from *E. gracilis* cells exhibited a significant content of Cys and GSH (Mendoza-Cózatl DG, Rangel-González EA, Moreno-Sánchez R, unpublished results). In a cell wall-deficient strain of *Chlamydomonas reinhardtii*, Cd^{2+} is also preferentially located inside the chloroplast and HMWC are also found in this organelle [46], but the origin of PC-like compounds and the mechanism by which Cd^{2+} is transported into the chloroplast are unknown [46]. *Chlorella* synthesizes PCs and forms HMWC in response to Cd^{2+} exposure, although the intracellular localization of these compounds is also unknown [47,48].

The preferential retention of Cd^{2+} over that of Zn^{2+} and Fe^{3+} in the *Euglena* chloroplast suggested that the accumulation process was specific for Cd^{2+}. This observation was in agreement with the notion that Cd^{2+} competes with Zn^{2+} in the uptake process by the chloroplast and that the chelating molecules involved in the metal retention, thiol-compounds and sulfide, have more affinity for Cd^{2+}.

The presence of PCs inside *E. gracilis* chloroplasts is intriguing. In plants and *Schizosaccharomyces pombe*, Cd-PC complexes are transported into vacuoles through an ABC-type transporter [25,26]. There are no reports about PCs transport into other organelles; in addition, PC synthase is thought to be a cytosolic soluble enzyme [49–51]. Then, the presence of PCs in *Euglena* chloroplast might be the result of any of the following processes:

(1) Active transport of PCs from cytosol to chloroplast.
(2) The existence of a PC synthase isoenzyme in *Euglena* chloroplast.
(3) Both processes coexist and *Euglena* can transport PCs and synthesize them inside the chloroplast.

Acknowledgements

This work was partially supported by CONACyT-México grant no. 43811-Q. PAEP grant no. 203319 (Facultad de Química, UNAM) and scholarship no. 144638 granted to DMC by CONACyT-México are also gratefully acknowledged.

References

[1] L.E. Williams, J.K. Pittman, J.L. Hall, Emerging mechanisms for heavy metal transport in plants, Biochim. Biophys. Acta 1465 (2000) 104–126.
[2] S. Clemens, Molecular mechanisms of plant metal tolerance and homeostasis, Planta 212 (2001) 475–486.
[3] J.L. Hall, Cellular mechanisms for heavy metal detoxification and tolerance, J. Exp. Bot. 53 (2002) 1–11.
[4] D.A. Cataldo, T.R. Garland, R.E. Wildung, Cadmium uptake kinetics in intact soybean plants, Plant Physiol. 73 (1983) 844–848.
[5] Y. Homma, H. Hirata, Kinetics of cadmium and zinc absorption by rice seedling roots, Soil Sci. Plant Nutr. 30 (1984) 527–532.
[6] G.L. Mullins, L.E. Sommers, Cadmium and zinc influx characteristics by intact corn (*Zea mays* L.) seedlings, Plant Soil 96 (1986) 153–164.

[7] J.M. Cutler, D.W. Rains, Characterization of cadmium uptake by plant tissue, Plant Physiol. 54 (1974) 67–71.
[8] J.J. Hart, W.A. Norvell, R.M. Welch, L.A. Sullivan, L.V. Kochian, Characterization of zinc uptake, binding, and translocation in intact seedlings of bread and durum wheat cultivars, Plant Physiol. 118 (1998) 219–226.
[9] J.J. Hart, R.M. Welch, W.A. Norvell, L.A. Sullivan, L.V. Kochian, Characterization of cadmium uptake, binding, and translocation in intact seedlings of bread and durum wheat cultivars, Plant Physiol. 116 (1998) 1413–1420.
[10] M. Solioz, C. Vulpe, CPx-type ATPases: a class of P-type ATPases that pump heavy metals, Trends Biochem. Sci. 21 (1996) 237–241.
[11] K.D. Hirschi, V.D. Korenkov, N.L. Wilganowski, G.J. Wagner, Expression of *Arabidopsis CAX2* in tobacco. Altered metal accumulation and increased manganese tolerance, Plant Physiol. 124 (2000) 125–133.
[12] P. Mäser, S. Thomine, J. Schroeder, J.M. Ward, K. Hirschi, H. Sze, I.N. Talke, A. Amtmann, F.J.M. Maathuis, D. Sanders, J.F. Harper, J. Tchieu, M. Gribskov, M.W. Persans, D.E. Salt, S.A. Kim, M.L. Guerinot, Phylogenetic relationships within cation transporter families of *Arabidopsis*, Plant Physiol. 126 (2001) 1646–1667.
[13] C. Curie, J.M. Alonso, M.L.E. Jean, J.R. Ecker, J.F. Briat, Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport, Biochem. J. 347 (2000) 749–755.
[14] S. Thomine, R. Wang, J.M. Ward, N.M. Crawford, J.I. Schroeder, Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 4991–4996.
[15] E.E. Rogers, D.J. Eide, M.L. Guerinot, Altered selectivity in an *Arabidopsis* metal transporter, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12356–12360.
[16] L.T. Paulsen, M.H. Saier, A novel family of ubiquitous heavy metal ions proteins, J. Membr. Biol. 156 (1997) 99–103.
[17] T. Bloß, S. Clemens, D.H. Nies, Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes, Planta 214 (2002) 783–791.
[18] T. Kambe, H. Narita, Y. Yamaguchi-Iwai, J. Hirose, T. Amano, N. Sugiura, R. Sasaki, K. Mori, T. Iwanaga, M. Nagao, Cloning and characterization of a novel mammalian zinc transporter, Zinc transporter 5, abundantly expressed in pancreatic β cells, J. Biol. Chem. 277 (2002) 19049–19055.
[19] N. Grotz, T. Fox, E. Connolly, W. Park, M.L. Guerinot, D. Eide, Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7220–7224.
[20] N.S. Pence, P.B. Larsen, S.D. Ebbs, D.L. Letham, M.M. Lasat, D.F. Garvin, D. Eide, L.V. Kochian, The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 4956–4960.
[21] S. Clemens, T. Bloss, C. Vess, D. Neumann, D.H. Nies, U.Z. Nieden, A transporter in the endoplasmic reticulum of *Schizosaccharomyces pombe* cells mediates zinc storage and differentially affects transition metal tolerance, J. Biol. Chem. 277 (2002) 18215–18221.
[22] C.W. MacDiarmid, L.A. Gaither, D. Eide, Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*, EMBO J. 19 (2000) 2845–2855.
[23] M.L. Guerinot, The ZIP family of metal transporters, Biochim. Biophys. Acta 1465 (2000) 190–198.
[24] D.E. Salt, G.J. Wagner, Cadmium transport across tonoplast of vesicles from oat roots, J. Biol. Chem. 268 (1993) 12297–12302.
[25] D.E. Salt, W.E. Rauser, MgATP-dependent transport of phytochelatins across the tonoplast of oat roots, Plant Physiol. 107 (1995) 1293–1301.
[26] D.F. Ortiz, T. Ruscitti, K.F. McCue, D.W. Ow, Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein, J. Biol. Chem. 270 (1995) 4721–4728.
[27] Z.-S. Li, Y.-P. Lu, R.-G. Zhen, M. Szczypka, D.J. Thiele, P.A. Rea, A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis (glutathionato) cadmium, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 42–47.
[28] R.K. Mehra, P. Mulchandani, T.C. Hunter, Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*, Biochem. Biophys. Res. Commun. 200 (1994) 1193–1200.
[29] D.M. Speiser, S.L. Abrahamson, G. Banuelos, D.W. Ow, *Brassica juncea* produces a phytochelatin-cadmium-sulfide complex, Plant Physiol. 99 (1992) 817–821.
[30] R.H. Juang, K.F. McCue, D.W. Ow, Two purine biosynthetic enzymes required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate in vitro, Arch. Biochem. Biophys. 304 (1993) 392–401.
[31] J.G. Vande, D.W. Ow, Accumulation of metal/binding peptides in fission yeast requires *hmt2*$^{+}$, Mol. Microbiol. 42 (2001) 29–36.
[32] D. Mendoza-Cózatl, S. Devars, H. Loza-Tavera, R. Moreno-Sánchez, Cadmium accumulation in the chloroplast of *Euglena gracilis*, Physiol. Plant. 115 (2002) 276–283.
[33] J.A. Schiff, H. Lyman, G.K. Russell, Isolation of mutants from *Euglena gracilis*, Methods Enzymol. 23 (1971) 143–162.
[34] T.J.M. Schoenmakers, G.J. Visser, G. Flik, A.P.R. Theuvenet, CHELATOR: an improved method for computing metal ion concentrations in physiological solutions, BioTechniques 12 (1992) 870–879.
[35] L.M. Sillen, A.E. Martell, Stability constants of metal-ion complexes, Spec. Publ. - Chem. Soc. 25 (1971) 865 pp.
[36] J.S. Rodríguez-Zavala, R. Moreno-Sánchez, Modulation of oxidative phosphorylation by Mg^{2+} in rat heart mitochondria, J. Biol. Chem. 273 (1998) 7850–7855.
[37] W.E. Rauser, Cadmium-binding peptides from plants, Methods Enzymol. 205 (1991) 319–333.
[38] T.E. King, R.O. Morris, Determination of acid-labile sulfide and sulfhydryl groups, Methods Enzymol. 10 (1957) 634–641.
[39] P.M. Hinkle, P.A. Kinsella, K.C. Osterhoudt, Cadmium uptake and toxicity via voltage-sensitive calcium channels, J. Biol. Chem. 262 (1987) 16333–163337.
[40] B. Fuks, F. Homblé, A voltage-dependent porin-like channel in the inner envelope membrane of plant chloroplasts, J. Biol. Chem. 270 (1995) 9947–9952.
[41] S. Clemens, D.M. Antosiewicz, J.M. Ward, D.P. Schachtman, J.I. Schroeder, The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 12043–12048.
[42] Z. Guan, B. Kuyoki, P. Feng, M.C. Kennedy, R.B. Franklin, L.C. Costello, Kinetic identification of a mitochondrial zinc uptake transport process in prostate cells, J. Inorg. Biochem. 97 (2003) 199–206.
[43] B.J. Van der Zaal, L.W. Neuteboom, J.E. Pinas, A.N. Chardonnens, H. Schat, J.A.C. Verkleij, P.J.J. Hooykaas, Overexpression of a novel *Arabidopsis* gene related to putative zinc-transporters genes from animals can lead to enhanced zinc resistance and accumulation, Plant Physiol. 119 (1999) 1047–1055.
[44] W.E. Rauser, P. Meuwly, Retention of cadmium in roots of maize seedlings, Plant Physiol. 109 (1995) 195–202.
[45] J.S. Wu, H.Y. Sung, R.H. Juang, Transformation of cadmium-binding complexes during cadmium sequestration in fission yeast, Biochem. Mol. Biol. Int. 36 (1995) 1169–1175.
[46] K. Nagel, U. Adelmeier, J. Voigt, Subcellular distribution of cadmium in the unicellular green alga *Chlamydomonas reinhardtii*, J. Plant Physiol. 149 (1996) 86–90.
[47] D. Kaplan, Y.M. Heimer, A. Abelovich, P.B. Goldsbrough, Cadmium toxicity and resistance in *Chlorella* sp, Plant Sci. 109 (1995) 129–137.

[48] W. Gekeler, E. Grill, E.L. Winnacker, M.H. Zenk, Algae sequester heavy metals via synthesis of phytochelatin complexes, Arch. Microbiol. 150 (1988) 197–202.
[49] S. Clemens, E.J. Kim, D. Neumann, J. Schroeder, Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast, EMBO J. 18 (1999) 3325–3333.
[50] O.K. Vatamaniuk, S. Mari, Y.-P. Lu, P.A. Rea, Mechanism of heavy metal ion activation of phytochelatin (PC) synthase, J. Biol. Chem. 275 (2000) 31451–31459.
[51] R. Ruotolo, A. Peracchi, A. Bolchi, G. Infusini, A. Amoresano, S. Ottonello, Domain organization of phytochelatin synthase, J. Biol. Chem. 279 (2004) 14686–14693.

De: BBA - Bioenergetics (ELS) <bbabio@elsevier.com>
Enviado el: Jueves, 24 de Junio de 2004 03:53:29 p.m.
Para: <cozatl@hotmail.com>
Asunto: BBABIO-04-2: Decision

Manuscript No.: BBABIO-04-2
Title: Cd2+ transport and storage in the chloroplast
of Euglena gracilis

Article Type: Regular Paper BBA Section: BBA - Bioenergetics
Corresponding Author: Dr. David Guillermo Mendoza-Cózatl
All Authors: David Guillermo Mendoza-Cózatl, PhD; Rafael Moreno-Sánchez, PhD.
Submit Date: Apr 07, 2004

Dear Dr. Mendoza-Cózatl:

On behalf of the Executive Editors of Biochimica et Biophysica Acta, I would like to thank you for submitting the above-mentioned article to BBA - Bioenergetics.

Your paper has been reviewed by three experts. One of these reviewers had somewhat minor comments and supported publication, the second reviewer raised some rather more important points, and the third reviewer was fairly negative. As a result, the manuscript is not acceptable in its present form.

However, because of the varied responses, I am willing to reconsider a substantially revised manuscript, provided that the major concerns of the second reviewer can be met, in particular:
--the need for better citation of literature;
--the need to describe growth media and chemical activity of cadmium in the growth media;
--and the possible effects of non-specific cadmium binding to the chloroplast surface. The more minor comments should also be addressed.

Please be advised that any resubmitted version of your article will be re-evaluated by one or more original reviewers.

When submitting your revised paper, please be sure to include a separate document uploaded as "Response to Reviewers" that carefully addresses, point-by-point, the issues raised in the comments appended below. You should also include a suitable rebuttal to any specific request for change that you have not made. Please note that the editorial policy of BBA places a time limit for resubmission of a revised manuscript at 6 months. Such a revised manuscript will retain its original submission date. Manuscripts submitted after 6 months will be considered as new submissions.

Yours sincerely,

Peter R. Rich, Ph.D.
Executive Editor
BBA - Bioenergetics
Please see Reviewers' comments below:

Reviewer #1: In this paper the authors have described experiments aimed at elucidating the mechanism of Cd2+ accumulation by chloroplasts from Euglena gracilis. This appears to occur by a mixture of simple and facilitated diffusion. Growth of the organism in the presence of Cd2+ leads to an increase in the storage capacity of chloroplasts for Cd2+ consistent with an increase in thiol compounds and sulphide in these chloroplasts as well as the occurrence of phytochelatins. The work is concisely and well described, except that the Tables in the manuscript do not correspond either with the text or the table legends.
In addition, Table IV (why the Roman numerals?) is missing data on the chloroplast sulphide content. On page 9, line 9, they state that the 'saturable component appeared with unacceptable huge dispersion of both Km and Vm values.'
However, they have included values for this condition in their table - there needs to be some indication of the reliability of these values in the table.
On page 11, line 8, they give values for Cd2+ release having already said on the previous page that 'Cd2+ release was too fast to allow an accurate estimation of release rate'. Given this, the difference in rate in the figures on page 11 is rather modest. This needs to be clarified or expanded upon.

There are other minor errors/comments:
1 Vm might be better replaced with Vmax. throughout
2 Page 4, line 9: suggest 'implicated in' rather than 'related to'
3 Page 12, line 14: omit 'it' after 'brought'
4 Page 13, second sentence of the Discussion: It is not clear what the logic of this statement is.
5 Page 13, 5th line from bottom: omit 'a' in 'In a previous work...'
6 Keywords: 'Cd2+' rather than 'C; 2+'

Reviewer #2: The paper submitted by Mendoza-Cozatl and Moreno-Sanchez describes the effects of various competing ions and inhibitors on cadmium uptake kinetics by isolated Euglena chloroplasts. In addition, the distribution of various Cd-thiol complexes in Euglena chloroplasts is described. The abstract and introduction are well written although references to the literature on phytochelatins synthase and algal heavy metal biology are incomplete and out of date.

The paper described two kinetically distinguishable cadmium uptake mechanisms, a saturable mechanism having classic Michaelis-Menten kinetics, and a non-saturable uptake system. The former system is competitively inhibited by zinc but not by other divalent metals suggesting similarity to a ZIP-type transporter. In addition, cadmium export from plastids is characterized, although the chemical form of the exported cadmium is not described.

There are several concerns regarding the paper that need to be addressed in any revised version:
1. The growth media and chemical activity of cadmium in the growth media are not described. It is unclear whether the effective concentration of cadmium in the medium is equivalent to that added.
2. Little care is taken to exclude the effects of non-specific cadmium binding to the chloroplast surface. This is most evident in the experiments described in Figure 3 that show a very rapid loss of cadmium from chloroplasts exposed to EGTA. The authors attribute this rapid loss of cadmium to a rapid export system. It is more likely due to removal of adventiously-bound cadmium from

the surface of the chloroplasts. It is suggested that a vast excess of a non-competing metal or "cold cadmium" be included in the export studies to displace any metal that may be bound non-specifically to the chloroplast surface.

Minor points:
1. The tables have values of mg-l but do not indicate what the identity of the mass is.
2. Page 11, line 3. This sentence is unclear and the values may be reversed for the two treatments.
3. Page 13, 2nd paragraph, line 4. Table III indicates rates of cadmium uptake not chloroplast cadmium or other metal content.

Overall, cadmium uptake, detoxification, and export from algal chloroplasts is a poorly understood mechanism. This research has the potential to contribute substantially to our understanding of the mechanisms by which algae tolerate toxic levels of heavy metals. It is recommended that the paper be substantially revised for publication.

Reviewer #3: The paper by Mendoza-Cózatl and Moreno-Sánchez deals with the process of Cd^{2+} uptake and storage into chloroplasts from Euglena gracilis. The scientific work conducted by the authors is sound and well described in the manuscript.
However, the objectives and addressed and questions dealt with are rather restricted and narrow. The novelty of the results and their relevance for the field at the molecular/mechanistic or cellular level are not obvious.
Therefore I feel that the manuscript in its present status should hardly be published.

INSTITUTO NACIONAL DE CARDIOLOGÍA
IGNACIO CHAVEZ

Mexico September 21, 2005

Dear Professor Rich:

Thank you for your kind Email of 24 June 2004 regarding the manuscript (BBABIO-04-2) entitled **"Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*"**. We were informed that a major revision of the manuscript addressing the observations made by the reviewers is required, before the paper may be considered for publication in BBA-Bioenergetics. In the enclosed revised manuscript, we have answered all the points raised by the reviewers. Accordingly, the manuscript was modified as described below; the numbering of the references also changed. We hope that in its present form the manuscript may be found acceptable for publication.

Sincerely

David G. Mendoza-Cózatl
Departamento de Bioquímica
Instituto Nacional de Cardiología
Juan Badiano # 1, Col. Sección XVI, Tlalpan 14080
México, DF. MÉXICO.
Fax: +5255-5573-0926
Email: cozatl@hotmail.com

Response to Reviewer #1

Major points

1. The tables changed to Arabic numerals and the correspondence between text and tables was checked and corrected.

2. Cd^{2+} uptake in the condition K^++ 4°C (Fig. 1B) was now fitted to a linear equation and the result was included in Table 1A. The previous *Vmax* and *Km* values with huge dispersion were deleted; the corresponding sentence in the text was also deleted.

3. Because the modest differences found in the Cd^{2+} release at the 10 s-point, in which conditions for measuring initial rate were not achieved, the previous sentence showing rates of Cd^{2+} release was deleted. New data about Cd^{2+} release were included in Fig. 3A and the text was modified in the Results (the three paragraphs of p. 13) and Discussion (p. 18, 2nd paragraph) sections.

Minor comments

1. Vm was replaced by *Vmax* throughout the text.

2. p. 4, line 9: "related in" was replaced by "implicated in".

3. p. 12, line 14: "it" was deleted.

4. p. 13, line 2: the sentence was rephrased.

5. p. 13, line5: "a" was deleted.

6. Keywords: "C;2+" was replaced by "Cd^{2+}" on the first page of the PDF file.

Response to Reviewer # 2

Major points

1. A detailed description of the growth medium and chemical activity of Cd^{2+} was included on p. 6, last paragraph and p. 7, 1st paragraph. Chemical activity of Cd^{2+} was estimated and experimentally determined at a pH of 3.5, the pH value of the culture medium, which changes by the eighth day of culture, when most of added Cd^{2+} has been accumulated (see Ref. Mendoza). The calculated values represent minimum values of free Cd^{2+} available in the culture media; the presence of the other metal ions should increase the free Cd^{2+} concentration. The free Cd^{2+} concentration was not determined in the complete culture medium because Mag-Fura-2 also reacts with the other metal ions, making the changes in the fluorescence signal difficult to interpret.

2. Certainly Cd^{2+} binds non-specifically to the chloroplast surface. A sentence stating this is included on p. 13, lines 13-15. However, bound cadmium was efficiently removed by washing the chloroplasts in the filters with $CdCl_2$, as stated in Material and Methods (p. 9, lines 4-6). Moreover, no differences were found by washing the chloroplasts with $CdCl_2$, EGTA, KCl or DTT (see please p. 13, last lines and p. 4, first two lines). This suggests that the main part of Cd^{2+} be localized inside the chloroplasts. As suggested by the reviewer, new data on cadmium release in the presence of competing and non-competitive ions were included on Fig 3A.

The chemical form in which cadmium is released is at the moment not known. This was stated on p. 14 lines 4-5. Updated information and references on phytochelatin synthase and algal heavy metal biology were added on p. 6, 1st paragraph; and p. 20, 1st paragraph.

Minor comments

1. The mass identity (mg protein^{-1}) was included in the Tables.

2. p. 11, line 3; The sentence was modified and expanded to clarify the idea.

3. There was an overlap in the Tables numbering. Table 3 now shows the cadmium, iron and zinc content in cells and chloroplasts exposed or not for eight days to 50 μM $CdCl_2$.

Response to Reviewer # 3

We have tried to present the objectives and questions analyzed in the present work in a more general context in the Introduction on p. 3-6 and in Discussion on p. 19-21. We think our research may contribute to the understanding of the cellular and molecular mechanisms involved in the resistance to toxic levels of Cd^{2+} and its associated accumulation in unicellular protists and probably other organisms. In addition, we would like to emphasize that this is the first work addressing the biochemical mechanism by which a non-essential and toxic heavy metal is transported and accumulated into the chloroplast and this is, precisely, the relevance of the work. We hope the reviewer may concur with our view.

De: BBA - Bioenergetics (ELS) <bbabio@elsevier.com>
Enviado el: Miércoles, 22 de Septiembre de 2004 06:05:14 p.m.
Para: <cozatl@hotmail.com>
Asunto: BBABIO-04-2R2: Final Decision

Manuscript No.: BBABIO-04-2R2
Title: Cd2+ transport and storage in the chloroplast
of Euglena gracilis

Article Type: Regular Paper
BBA Section: BBA - Bioenergetics
Corresponding Author: Dr. David Guillermo Mendoza-Cózatl
All Authors: David Guillermo Mendoza-Cózatl, PhD; Rafael Moreno-Sánchez, PhD
Submit Date: Apr 07, 2004

Dear Dr. Mendoza-Cózatl:

We are pleased to inform you that the above-named manuscript has been accepted for publication in Biochimica et Biophysica Acta. Your article has been forwarded to Elsevier's Production Department in Amsterdam and will be included for publication in BBA - Bioenergetics.

Your article will be published rapidly in electronic form, as well as in the traditional print journal in the first available scheduled issue.

Shortly, you will receive an acknowledgement letter from our Production Department detailing information regarding proofs, reprints and copyright transfer. The BBA Editorial Office handles only editorial matters and does not have any information regarding production issues. Should you have any further inquiries regarding this manuscript, please contact our author support department at: authorsupport@elsevier.com

We once again thank you for your contribution to BBA - Bioenergetics and hope that you will continue to submit your research articles to the journal.

On behalf of Dr. Peter Rich,
Yours sincerely,

Laura Wallins, D.Sc.
Editorial Office
BBA - Bioenergetics

6.1.1 Datos no mostrados en el trabajo "Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*"

La gráfica 6.1.1 muestra los dobles recíprocos del transporte de Cd^{2+} en cloroplastos control. Se puede distinguir una desviación de la linealidad, la cual puede interpretarse como: (a) dos componentes o bien, (b) cooperatividad negativa. La curva de saturación (v vs Cd^{2+}; ver Fig 1A de Mendoza-Cózatl y Moreno-Sánchez R, 2005) no muestra la tendencia característica de una cooperatividad negativa. Por otro lado, no se obtuvo un buen ajuste de los datos utilizando la ecuación de Hill (Tabla 6.1.1), la cual es capaz de describir la cooperatividad negativa. Estos resultados apoyaron la presencia de dos componentes en el transporte de Cd^{2+} al cloroplasto de *Euglena gracilis*.

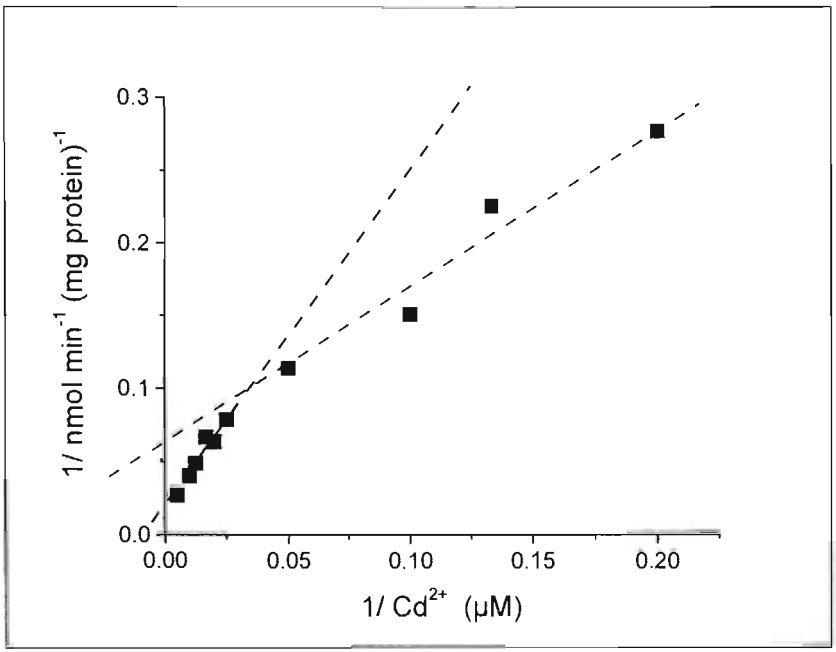


Figura 6.1.1 Dobles recíprocos del transporte de Cd^{2+} en cloroplastos control (25°C)

La tabla 6.1.1 muestra los resultados de los ajustes del transporte de Cd^{2+} a diferentes ecuaciones, incluida la usada en la Fig. 1A del transporte de Cd^{2+} al cloroplasto (ver Fig 1A de Mendoza-Cózatl y Moreno-Sánchez R, 2005). Hay que puntualizar que la Chi^2 no puede ser usada en este caso como única referencia para evaluar los ajustes, debido a que las ecuaciones contienen un número distinto de parámetros. Los resultados, ponderando la desviación de los parámetros cinéticos, muestran que el mejor ajuste se logra con una ecuación que involucra dos componentes, uno saturable e hiperbólico y otro lineal no-saturable.

Tabla 6.1.1 Parámetros cinéticos de diferentes ajustes por regresión no-lineal realizados al transporte de Cd^{2+} en cloroplastos control (ver Fig 1A de Mendoza-Cózatl y Moreno-Sánchez R, 2005).

	MM[a] + línea	doble MM	Hill	doble Hill
	Chi^2 1.04	Chi^2 0.91	Chi^2 1.03	Chi^2 0.74
	Vm 10.5 ± 2[b]	*Vm 1* 107 ± 48	*Vm* 387 ± 921	*Vm 1* 23.8 ± 25
Cloroplastos	*Km* 13.9 ± 6.1	*Km 1* 468 ± 351	$K_{0.5}$ 5629 ± 24451	$K_{0.5}$ *1* 34 ± 80
control	*m* 0.13 ± 0.1	*Vm2* 4.9 ± 3.4	*h* 0.67 ± 0.09	*n1* 0.8 ± 0.5
		Km2 3.9 ± 6.8		*Vm2* 18 ± 14
				$K_{0.5}$ *2* 107 ± 10
				n2 4.4 ± 3.2

[a] Michaelis-Menten
[b] La dispersión de la media se refiere al error estándar derivado del ajuste de los datos experimentales a la ecuación respectiva y, por tanto, no tiene que ver con el número de experimentos.

La tabla 6.1.2 muestra que el rojo de rutenio, mersalyl, la luz o la oscuridad no tienen efecto sobre el transporte de Cd^{2+} en cloroplastos de *Euglena gracilis*, por lo que el

transporte depende únicamente de la concentración de Cd^{2+} a ambos lados de la membrana del cloroplasto.

Tabla 6.1.2 Efecto del rojo de rutenio, mersalyl, luz y oscuridad en el transporte de $^{109}Cd^{2+}$ en cloroplastos control

	Rojo de rutenio (50 µM)	Mersalyl (50 µM)	Luz*	Oscuridad
		nmoles $^{109}Cd^{2+}$ min^{-1} mg $proteína^{-1}$		
Cloroplastos Control	21.4 ± 4.6 (4)	20 ± 3.4 (3)	19.5 ± 2.7 (3)	23 ± 4 (3)

*Los cloroplastos se iluminaron 60 segundos antes de iniciar el experimento del transporte y se continuó durante el experimento. Los experimentos se realizaron con 100 µM $^{109}CdCl_2$. Promedio ± DE (n).

Una de las posibilidades para explicar el aumento en la velocidad de captación de Cd^{2+} hacia el cloroplasto de *Euglena* (ver tabla 1B de Mendoza-Cózatl y Moreno-Sánchez R, 2005), era un cambio inespecífico en la relación proteína/clorofila. Una disminución en la concentración de proteína a una concentración constante de clorofila, produciría un incremento en la velocidad del transporte si se usa como única referencia la cantidad de proteína en el ensayo. La tabla 6.1.3 muestra que esta relación permanece constante, por lo que el aumento en la *Vm* del transporte podría deberse a un aumento en la cantidad de transportador en la membrana del cloroplasto.

Tabla 6.1.3 Relación proteína/clorofila en células control y cultivadas con 50 µM $CdCl_2$

Condición de cultivo	Relación proteína/clorofila (mg proteína·$(10^7 cels)^{-1}$ / µg clotrofila·$(10^7 cels)^{-1}$
Control	0.11 ± 0.02 (5)*
ZCd_{50}	0.11 ± 0.01 (5)

*Promedio ± DE (n).

La figura 6.1.2 muestra el gráfico de Dixon (1/v vs inhibidor [Zn^{2+}]) para determinar la *Ki* del transportador (componente saturable) de Cd^{2+} hacia el Zn^{2+} en cloroplastos de *E. gracilis* (ver Fig. 2B en Mendoza-Cózatl y Moreno-Sánchez R, 2005).

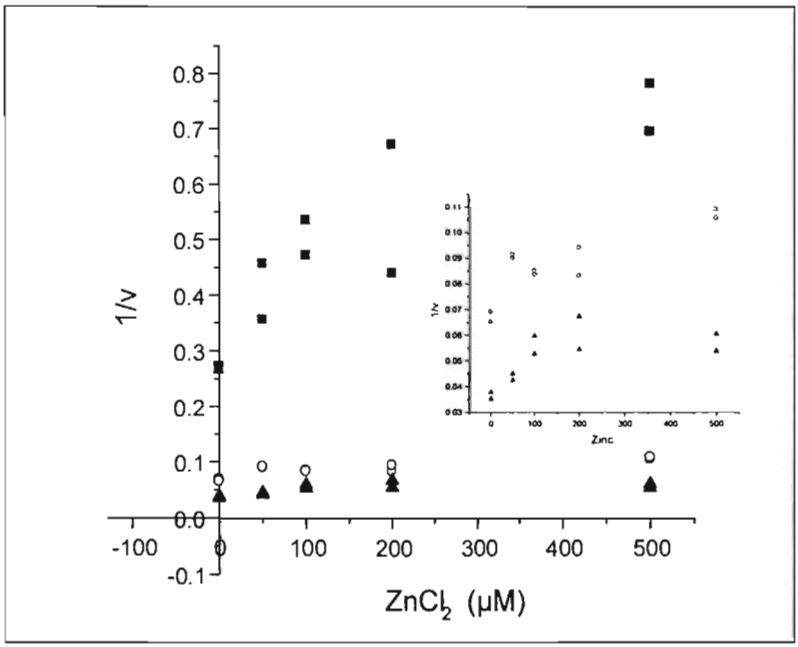


Figura 6.1.2 Gráfico de Dixon para la inhibición del transporte de Cd^{2+} por Zn^{2+}.

Las concentraciones de Cd^{2+} usadas fueron: 10 μM (■), 50 μM (○) y 100 μM (▲). El inserto es una amplificación de las concentraciones 50 y 100 μM Cd^{2+}.

La tabla 6.1.4 muestra los ajustes por regresión no-lineal de la inhibición del transporte de Cd^{2+} de cloroplastos de *Euglena* por Zn^{2+}. Una vez más, basándose en las desviaciones estándar de los ajustes, podemos concluir que la inhibición del Zn^{2+} es de tipo competitiva.

Tabla 6.1.4 Ajustes por regresión no-lineal del tipo de inhibición del transporte de Cd^{2+} en cloroplastos.

	Competitivo	No competitivo	Incompetitivo	Mixto
Cloroplastos control	Chi^2 3.01 *Ki* 8.5 ± 2 µM	Chi^2 3.56 *Ki* 50.3 ± 12.5	Chi^2 4.03 *Ki* 30 ± 10	Chi^2 3.04 *Ki* 4.7 ± 2.9 α -0.07 ± 0.04

6.2 Consideraciones termodinámicas del transporte de Cd^{2+} en cloroplastos de *Euglena gracilis*

p 82-84 Problema Bioquímico REB 22:86, 92-93

PROBLEMA BIOQUÍMICO II

David Mendoza Cózatl y Rafael Moreno Sánchez
Correo E: davidmendozac@netscape.net

ioenergética
ransporte de cadmio en cloroplasto

l cadmio es un metal pesado *no esencial*, el cual es tóx-:o para la mayoría de los organismos. *Euglena gracilis* es n protista fotosintético con una elevada capacidad de tol-rancia y acumulación de cadmio, y la mayor parte del ietal es acumulado en el cloroplasto. Para determinar la inética de captación, y el mecanismo por el cual el cad-nio es acumulado en el cloroplasto, se obtuvieron las elocidades iniciales de captación de cadmio en cloro-lastos intactos de *Euglena gracilis*. Además, se evaluó el fecto de la temperatura y de diferentes inhibidores CCCP, desacoplante y DCMU, inhibidor del Fotosistema I) sobre la velocidad de captación, para determinar a que

Tabla 1 Velocidades iniciales de transporte de cadmio en cloroplastos intactos de *Euglena gracilis* a diferentes temperaturas.

$CdCl_2$ (μM)	25 °C	4 °C
	nmol Cd min^{-1} mg^{-1}	
0	0	0
5	3.61	2.9
7.5	4.43	n.d.
10	6.62	4.12
15	7.73	n.d.
20	8.8	5.21
50	15.69	6.86
75	19.59	8.1
100	24.72	10.3
150	29.74	13.1
200	36.88	16.41

n.d. no determinada

Tabla 2 Efecto de inhibidores sobre el transporte de cadmio en cloroplastos de *Euglena gracilis*

Cloroplastos control (100 μM)	(100 μM $CdCl_2$) + DCMU	+ CCCP (50 μM)
nmol Cd min^{-1} mg^{-1}		
21.4 ± 4.6 (8)	19.4 ± 3 (3)	19 ± 4.2 (4)

promedio ± DE (n)

tipo de energía está acoplado el transporte.

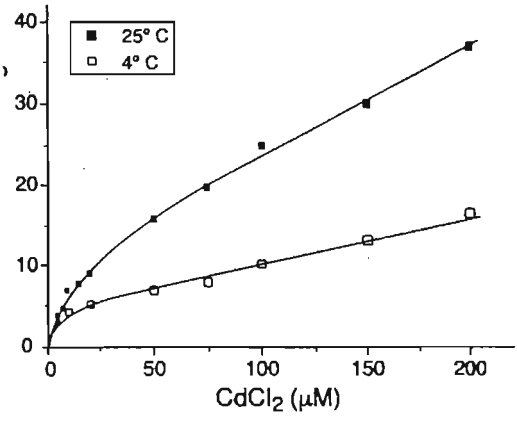
1.- Determinar los parámetros cinéticos (*Km* y *Vm*) de el o los componentes involucrados en el transporte de cadmio.

2.- ¿A que tipo de energía esta acoplado el transporte?

3.- Si la concentración máxima de cadmio libre en el citosol es de 10 μM y la de el cloroplasto es 5 μM, y durante la fotofosforilación se establece una diferencia de potencial eléctrico transmembranal ($\Delta\Psi$) entre el citosol y el estroma de −100 mV (interior negativo), calcule el cambio de energía libre (ΔG_{Cd}) para el transporte de cadmio del citosol al cloroplasto a 25 °C. ¿Es termodinámicamente favorable este proceso?

RESPUESTA AL PROBLEMA BIOQUÍMICO II

l graficar la velocidad de transporte contra la concen-ıción de cadmio se obtienen a ambas temperaturas, :rvas que no se ajustan a una hipérbola (cinética de ichaelis-Menten) (Fig. 1). La gráfica de dobles cíprocos muestra curvas convexas (Fig. 2). Estos sultados pueden interpretarse de dos maneras: (1) dos mponentes están actuando simultáneamente en el nsporte o bien, (2) una sola enzima presenta cooper-ividad negativa (una curva cóncava se interpretaría mo cooperatividad positiva). La gráfica de velocidad ntra sustrato a 4 °C define con mayor claridad dos mponentes, de los cuales uno es completamente line- sugiriendo que parte del transporte se lleva acabo por fusión simple. La curva de saturación presente a bajas ncentraciones de cadmio, y claramente visible a °C, sugiere que la parte restante del transporte es ediado por una proteína.



gura 1

La *Vm* del componente saturable (18.8 nmoles Cd min ng⁻¹ a 25 °C), obtenida de la zona lineal en los dobles cíprocos a bajas concentraciones de Cd, será una *Vm* arente (Vm_{app}) pues se encuentra modificada por el mponente lineal. Sin embargo, esta Vm_{app} nos indica e por arriba de esta velocidad, el transporte es única-nte debido al componente lineal (difusión). De hecho, a regresión lineal con los datos de la Fig. 1 a 75, 100, 0 y 200 μM de $CdCl_2$ tiene un coeficiente de cor-ación de 0.995 (recuadro de la figura 3), y el valor de pendiente (0.140) nos indica cuanto aumenta la veloci-d de difusión por unidad de tiempo por cada μmol de l añadido L⁻¹.

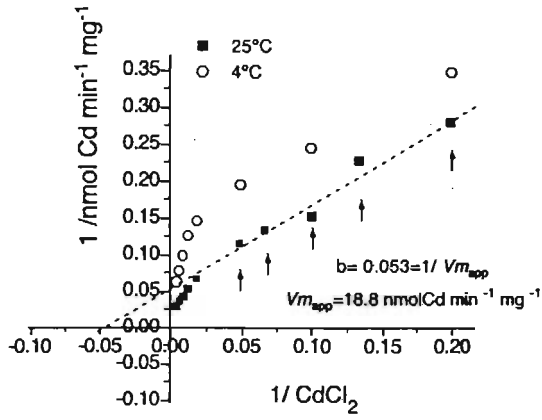


Figura 2

Conociendo la velocidad de difusión, y debido a que esta depende únicamente de la concentración de Cd añadido, la velocidad de transporte del componente saturable puede obtenerse restándole a la velocidad total la velocidad de difusión. Esta se obtiene al multiplicar la pendiente del componente lineal (0.140 nmol Cd min^{-1} mg^{-1} μM^{-1}) por la concentración a la cual se ensayó el transporte (Fig. 3). De los dobles recíprocos de las velocidades corregidas (Fig. 4) podemos obtener los valores reales de las constantes cinéticas para el componente saturable (Vm = 11 nmol Cd min^{-1} mg^{-1} y Km = 14.28 μM).

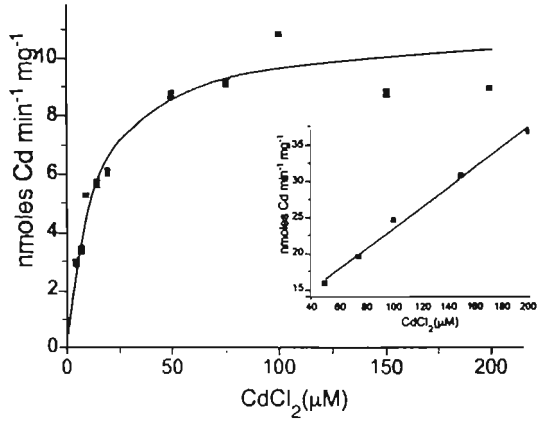


Figura 3

Los valores obtenidos por regresión no lineal, usando el programa Microcal Origin 5.0, al ajustar las velocidades iniciales de la Fig. 1 usando la ecuación $v = (Vm*S / Km + S) + (m*S)$, concuerdan con los valores obtenidos por regresión lineal.

Por otro lado, que el transporte de Cd no haya sido afectado por un desacoplante (CCCP) o un inhibidor de la fotosíntesis (DCMU), indica que el transporte no esta asociado a un potencial electroquímico de protones y que no depende de la actividad de los fotosistemas. Debido a que una proteína esta involucrada, estos resultados sug-

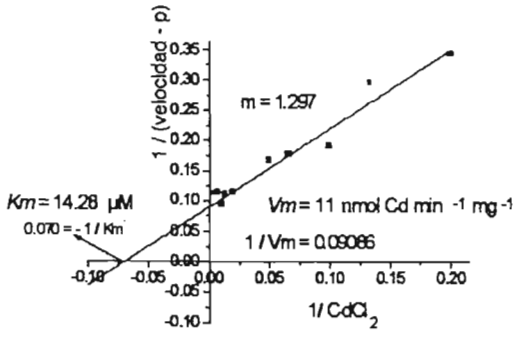


Figura 4

ieren que parte del cadmio entra al cloroplasto por difusión facilitada.

Finalmente, usando la ecuación $\Delta G_{Cd} = RT\ln ([Cd]_{dentro} / [Cd]_{fuera}) + Z_{Cd} F \Delta\Psi$, donde R = 1.98 cal mol^{-1} K^{-1}, T = 298.15 (25°C), Z_{Cd} = 2 (carga del ion), F = 23 080 cal mol^{-1} V^{-1} (Cte. de Faraday) y $\Delta\Psi$ = - 0.1 V (potencial de membrana) tenemos que:

$$\Delta G_{Cd} = 1.987 \times 298.15 \ln (5 \times 10^{-6} M / 10 \times 10^{-6} M) + [2 \times (23\ 080) \times (-0.1)]$$

durante la fotofosforilación, el potencial electroquímico del cadmio es ΔG_{Cd} = - 5.02 kcal mol^{-1}, por lo que el transporte de cadmio es termodinámicamente favorable.

REFERENCIAS.

Hart JJ, Welch RM, Norvell WA, Sullivan LA, Kochian LV (1998) Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiol 116 : 1413-1420.

Voet D and Voet JG (1990) Transporte a través de membranas. En : Bioquímica. Ed. OMEGA, Barcelona, España. pp. 520-544.

El *problema bioquímico* presentado ayuda a responder la pregunta ¿Que impulsa la entrada de Cd^{2+} al cloroplasto de *Euglena gracilis*? De acuerdo con la ecuación para calcular el potencial electroquímico del Cd^{2+} (ΔG_{Cd} ; Ecuación 1), la entrada de Cd^{2+} al cloroplasto estaría termodinámicamente favorecida durante la fotofosforilación (- 5.02 kcal mol^{-1}), si la concentración de Cd^{2+} en el citosol es mayor que en el estroma. Sin embargo, la entrada de Cd^{2+} al cloroplasto fue la misma con iluminación o en la oscuridad (ver capitulo 6.1). De modo que la pregunta sigue siendo válida, durante la oscuridad ¿El transporte de Cd^{2+} estaría igualmente favorecido termodinámicamente?.

$$\Delta G_{Cd} = RT\ln([Cd^{2+}]_{estroma}/[Cd^{2+}]_{citosol}) + Z_{Cd}F\Delta\psi \qquad \text{(Ecuación 1)}$$

En la oscuridad, la diferencia de potencial eléctrico entre el citosol y el estroma puede disminuir significativamente, por lo que la contribución del segundo término de la ecuación 1 (el componente eléctrico) al valor global del ΔG_{Cd} disminuye; sin embargo, no llega a cero o adquiere valor positivo debido a las ATPasas que se encuentran en la membrana externa del cloroplasto, las cuales bombean H^+ hacia el citosol manteniendo una diferencia mínima de potencial eléctrico entre el estroma y el citosol necesario para el transporte de solutos (Heiber et al., 1995). Por otro lado, aún sin una diferencia de potencial, la ecuación 1 también muestra que en cualquier condición en la que la concentración de Cd^{2+} fuera del cloroplasto sea mayor que en el estroma, y por lo tanto el componente químico $\ln([Cd]_{estroma}/[Cd]_{citosol})$ adquiera un valor negativo, el transporte de Cd^{2+} hacia el cloroplasto estaría favorecido termodinámicamente. Adicionalmente, hay que considerar que si dentro del cloroplasto hay moléculas capaces de secuestrar al Cd^{2+}, el transporte hacia el interior estaría aún más favorecido.

Es notable, sin embargo, que la diferencia del ΔG_{Cd} entre luz y oscuridad pueda ser de un orden de magnitud o más. Por ejemplo, asumiendo que la concentración de Cd^{2+} en el citosol y el estroma sea igual ($[Cd]_{estroma} = [Cd]_{citosol}$), el primer término de la ecuación 1 se elimina debido a que *ln* 1 = 0, por lo que el ΔG_{Cd} depende únicamente del segundo término. Tomando como ejemplo dos valores de diferencia de potencial -0.1 V (luz) y -0.01 (oscuridad), el ΔG_{Cd} sería -4.6 y -0.46 kcal mol^{-1}, respectivamente. ¿Que tanto podría influir esta diferencia en la velocidad de transporte? La respuesta es que podría o no podría influir.

Una reacción catalizada por una enzima está gobernada por dos factores los cuales pueden ser claramente separados, un término cinético, el cual tiene que ver con la k_{cat} ó constante catalítica de la enzima y la afinidad de la enzima por los sustratos o productos respectivos (Km_S *y/o* Km_P) y un segundo término, denominado termodinámico, el cual considera la constante de equilibrio de la reacción (K_{eq}) y por lo tanto el ΔG, ya sea el valor estándar o de preferencia el fisiológico (Ecuación 2).

$$v = \left(\frac{V_f}{Ks} \bullet \frac{1}{1 + s/Ks + p/Kp} \right) \bullet \left(s - \frac{p}{Keq} \right) \qquad \text{(Ecuación 2)}$$

Una reacción puede entonces estar gobernada cinéticamente, termodinámicamente o un balance de ambas. Siguiendo con el ejemplo anterior, un cambio de un orden de magnitud en el ΔG_{Cd} representa un cambio de 1000 veces en la *Keq* de la reacción, 2.16 para el caso de 0.01 V y 2230 para el caso de 0.1 V. Para demostrar cuanto puede variar la velocidad debido a un cambio en la *Keq* de 1000 veces, vamos a considerar que solamente el componente termodinámico afecta la velocidad de la reacción, por lo que la ecuación 2 quedaría simplificada a la ecuación 3:

$$v = 1 \bullet 1 \bullet \left(s - \frac{p}{Keq} \right)$$ (Ecuación 3)

Cuando s = p, un cambio en la constante de equilibrio de 2.16 a 2230 aumentaría la velocidad de la reacción 1.86 veces. Sin embargo, en condiciones de *velocidad inicial*, tal y como las utilizadas en el transporte de Cd^{2+} en cloroplastos de *Euglena*, cuando P es igual o muy cercano a cero, el cambio es mínimo, porque el término p/*Keq* es muy pequeño, si no es que despreciable. Por lo tanto, en una reacción catalizada enzimáticamente, el término termodinámico es importante para indicarnos hacia que dirección se llevará a cabo la reacción, hacia adelante (signo +) o en dirección reversa (signo -).

En conclusión, el cambio de mil veces en la *Keq* no modificó la velocidad de entrada de Cd^{2+} al cloroplasto debido a que los ensayos se llevaron a cabo en condiciones de *velocidad inicial*, donde la velocidad esta principalmente determinada por la capacidad catalítica del transportador, *Vm* ó k_{cat} * $[E_T]$, y más específicamente por su eficiencia catalítica (Vm / K_m).

6.3 Síntesis de fitoquelatinas e inactivación del Cd^{2+} en el cloroplasto de *Euglena gracilis* *

Una vez que determinamos que el Cd^{2+} y las FQs se acumulan principalmente en el cloroplasto, que el Cd^{2+} puede entrar al cloroplasto como ion libre y que son los tioles orgánicos (Cys, GSH, FQs) y el sulfuro las moléculas que lo retienen dentro del cloroplasto (ver capitulo 6.1, Mendoza-Cózatl y Moreno-Sánchez R, 2005), el siguiente paso fue determinar si las FQs podían sintetizarse en el interior del cloroplasto o si estas tenían que ser exclusivamente transportadas del citosol al cloroplasto.

En plantas y en la levadura *S. pombe*, las FQs pueden ser transportadas activamente del citosol a la vacuola a través de un transportador de tipo ABC (**A**TP **B**inding **C**assette; ver capitulo 2). Esta es la principal razón por la cual en la literatura se sugiere que la fitoquelatina sintetasa es un enzima citosólica; sin embargo, ningún trabajo ha determinado sistemáticamente su localización intracelular.

Utilizando cloroplastos purificados de *Euglena*, con poca o nula contaminación por citosol (< 10%), se determinó que las FQs pueden sintetizarse en el interior del cloroplasto y el porcentaje de la enzima total que se encuentra en el interior de este organelo. Por otro lado, un proceso clave en la inactivación del Cd^{2+} en plantas y levaduras como *Candida glabrata* y *S. pombe* es la formación de complejos de alto peso molecular formados por tioles, Cd^{2+} y sulfuro (ver capitulo 2), por lo que también se determinó si estos complejos se pueden formar dentro del cloroplasto de *Euglena gracilis*.

* **En este trabajo participaron: Mendoza-Cózatl D y Moreno-Sánchez R.**

Material y Métodos

Aislamiento de cloroplastos y obtención de citosol

Los cloroplastos se purificaron a partir de células en la fase estacionaria temprana de cultivo (8 días), expuestas o no a 50 μM de $CdCl_2$ (cloroplastos ZCd_{50}) de igual manera a lo reportado anteriormente (Mendoza-Cózatl y Moreno-Sánchez, 2005) con algunas modificaciones, las cuales se indican a continuación. Una vez rotas las células por sonicación, el extracto se centrifugó a 200 x *g* por 5 min para sedimentar células enteras; el sobrenadante se centrifugó a 2000 x *g* por 5 min para sedimentar los cloroplastos y el sobrenadante de esta fracción se centrifugó a 225 000 x *g* por 45 min para sedimentar la fracción microsomal. El sobrenadante de esta última fracción fue considerado como el citosol. La pastilla correspondiente a la centrifugación de 2000 x *g* enriquecida con cloroplastos se resuspendió y se aplicó a un gradiente discontinuo de Percoll (40-80%). Después de centrifugar a 6500 x *g* por 20 min, los cloroplastos intactos se localizaron en la interfase; estos cloroplastos fueron diluidos (20:1) y lavados dos veces con un medio libre de EDTA o buffer SH (0.33 M Sorbitol, HEPES 10 mM pH 7.3;) y finalmente resuspendidos en un volumen mínimo de buffer SH.

Para obtener las proteínas del estroma, los cloroplastos intactos se diluyeron 10 veces con Tris 20 mM pH 8, previamente burbujeado con nitrógeno, y se sometieron a 3 ciclos de congelación con nitrógeno líquido y descongelamiento (37 °C). El extracto se centrifugó a 20 000 x *g* por 10 min y el sobrenadante se consideró como el estroma. Antes de usarse, todos los buffers fueron previamente saturados con N_2. La proteína se determinó de acuerdo al método de Biuret usando BSA como estándar y centrifugando la mezcla para eliminar la interferencia del paramilo. La clorofila se extrajo con acetona al 80% y se cuantificó de acuerdo a lo reportado por Arnon (1949).

Actividad de fitoquelatina sintetasa (PCS), gliceraldehido 3-fosfato deshidrogenasa dependiente de NADP$^+$ (GAPDH-NADP$^+$) y glutamato deshirogenasa (GDH)

Para eliminar compuestos de bajo peso molecular incluidos sustratos y cofactores que pudieran interferir con el ensayo de la PCS, todas las fracciones (extracto crudo, estroma y citosol) se pasaron a través de una columna de Sephadex G25 y el eluado se concentró por ultra-filtración con un filtro Amicon Ultra (30 kDa). Todos los buffers usados fueron previamente saturados con N_2.

El ensayo de la PCS se realizó en Tris-HCl 200 mM pH 8.0, DTT (DL-ditiotreitol) 1 mM, GSH 1 mM. La reacción se inició con la adición del extracto (1-3 mg proteina/ml). A diferentes tiempos (0-60 min), alícuotas de la mezcla de reacción (450 µl) se acidificaban con ácido perclórico 3 % final, se centrifugaba a 20 000 x *g* para descartar la proteína precipitada y se analizaba el contenido de fitoquelatinas por RP-HPLC.

La GAPDH-NADP$^+$ se determinó por el consumo de NADPH (ε = 6220 M^{-1} cm^{-1} a 340 nm) en Tris 100 mM pH 7.8, $MgCl_2$ 10 mM, GSH 5 mM, ATP 5 mM, 0.2 unidades de 3-fosfoglicerato cinasa, 0.2 mM NADPH; después de una incubación de 4 min, la reacción se iniciaba añadiendo 3-fosfoglicerato 2 mM concentración final (Latzko y Gibbs, 1969).

La GDH se midió por la formación de NADPH en Tris 50 mM pH 8.0, NADP$^+$ 0.2 mM. Después de la adición del extracto (1-3 mg proteína), la reacción se iniciaba con la adición de Glu 30 mM (Suzuki et al., 1987).

Obtención de fracciones de alto (>50 kDa) y bajo peso molecular del estroma

Los cloroplastos obtenidos de células cultivadas con 50 µM $CdCl_2$ y purificados con Percoll (8-10 mg; 500µl) se solubilizaron con 0.2% de Tritón X-100 (concentración final), se sometieron a 3 ciclos de congelación-descongelación, con nitrogeno liquido y un baño a

30°C, respectivamente, y se centrifugaron por 20 min a 20 000 x *g*. El sobrenadante se fraccionó con un Centricón (50 kDa). El concentrado se resuspendió en Tris 50 mM (pH 8.0) y se concentró por segunda vez. Ambas fracciones se analizaron por RP-HPLC, además de medir el contenido de Cd^{2+} tal y como se reportó previamente (Mendoza-Cózatl y Moreno-Sánchez R, 2005).

Resultados y Discusión

Síntesis de fitoquelatinas en el cloroplasto de Euglena gracilis

Los cloroplastos control de *E. gracilis* fueron capaces de sintetizar FQs usando el GSH endógeno cuando se les expuso a Cd^{2+} 500 µM por una hora (Fig. 6.3.1). Esta observación muestra que, al igual que la enzima reportada para plantas y *S. pombe*, la PCS de los cloroplastos control de *E. gracilis* es una enzima constitutiva. La tabla 6.3.1 muestra el contenido de tioles solubles en ácido antes y después de la exposición a Cd^{2+}. Los únicos dos compuestos que varían significativamente durante la exposición a Cd^{2+} *in vitro* son la PC_2 y la PC_3, y la diferencia respecto a los cloroplastos incubados sin Cd^{2+} nos permitió calcular una velocidad de síntesis de 150 pmoles –SH (mg proteina)$^{-1}$ h^{-1}. Debido al alto contenido de FQs presentes en los cloroplastos obtenidos de células cultivadas con Cd^{2+} (cloroplastos ZCd_{50}), la síntesis *de novo* de FQs no pudo ser detectada (Tabla 6.3.1).

Para determinar la distribución intracelular de la PCS en *Euglena* y debido a la baja actividad de la enzima, fue necesario hacer un extracto enriquecido del estroma y eliminar la mayor parte de las FQs endógenas mediante una columna de exclusión molecular (Sephadex G25). A pesar de que este procedimiento no eliminó la totalidad de la PC_2 endógena, sí permitió determinar con mayor confianza la velocidad inicial para la PCS de *Euglena gracilis*, la cual fue lineal por 60 min (Figura 6.3.2). El mismo procedimiento

permitió medir la actividad en el extracto crudo (sonicado) y citosol (Tabla 6.3.2). La tabla 6.3.2 también muestra la actividad de la GAPDH-$NADP^+$ y GDH, enzimas marcadoras de estroma y citosol, respectivamente (Buetow, 1989), con el fin de determinar qué porcentaje de la PCS total está presente en el cloroplasto de *Euglena*.

Con la actividad de la PCS en el extracto crudo y en el cloroplasto purificado, más la cantidad de clorofila por célula (55 µg Chl/10^7 células) y la relación mg proteína/µg Chl en cada fracción, pudimos determinar que el 39% de la actividad de la PCS se encuentra en el cloroplasto (Tabla 6.3.2). Es importante notar que los cloroplastos no presentaron contaminación significativa por el citosol (actividad nula de la GDH), el cual sería la única fuente de contaminación para la actividad de PCS. Por otro lado, la elevada actividad de la GAPDH-$NADP^+$ encontrada en el citosol sugiere que una gran cantidad de cloroplastos se rompieron durante el proceso de sonicación; sin embargo, esto no afecta el cálculo del porcentaje de PCS en el cloroplasto debido a que la actividad se midió también en cloroplastos intactos después de ser purificados por un gradiente de Percoll. El hecho de haber encontrado una PCS en el interior del cloroplasto tiene implicaciones filogenéticas muy importantes, las cuales se discutirán en el apartado siguiente (ver capitulo 6.4).

Distribución de tioles en complejos de alto y bajo peso molecular en el cloroplasto de Euglena gracilis

Uno de los procesos clave para la inactivación y almacenaje del Cd^{2+} es la formación de complejos de alto peso molecular formados por tioles, Cd^{2+} y sulfuro; en plantas y algunas levaduras, la incapacidad de formar estos compuestos resulta en una hipersensibilidad a Cd^{2+} (Vande y Ow, 2000). Si la acumulación de Cd^{2+} en el cloroplasto

de *Euglena* esta relacionada con el mecanismo de resistencia, entonces se esperaria encontrar este tipo de complejos dentro del cloroplasto de *Euglena*.

El fraccionamiento del estroma de cloroplastos ZCd_{50} en compuestos de alto (>50 kDa) y bajo (<50 kDa) peso molecular permitió determinar que parte del Cd^{2+}, sulfuro y FQs, los cuales son péptidos de muy bajo peso molecular (< 2 kDa), se encontraban en la fracción de **alto** peso molecular (> 50 kDa, Fig. 6.3.3). La tabla 6.3.3 muestra que más del 70% de las FQs se encuentran en la fracción de alto peso molecular, mientras que monotioles como γ-EC y GSH se encuentran principalmente en la fracción de bajo peso molecular. Estos resultados sugieren que los complejos tiol- Cd^{2+}-sulfuro de alto peso molecular están relacionados con la acumulación e inactivación del Cd^{2+} en el cloroplasto de *Euglena gracilis*.

Referencias

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Plant Physiol 24: 1–15

Buetow DE (1989) The biology of *Euglena*. Buetow DE (ed.), vol IV Academic press, Inc. 511 pp. ISBN 0-12-139904-4.

Latzko E, Gibbs M (1969) Enzyme activities of the carbon reduction cycle in some photosynthetic organisms. Physiol. Plant 44:295-300.

Mendoza-Cózatl D, Moreno-Sánchez R. (2005) Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*. Biochim. Biophys. Acta 1706: 88-97.

Suzuki, E, Tsuzuki M, Miyachi S (1987) Photosynthetic characteristics of chloroplasts isolated from *Euglena gracilis* Z grown photoautotrophycally. Plant Cell Physiol. 28:1377-1388.

Vande WJG, Ow DW (2001) Accumulation of metal-binding peptides in fission yeast requires hmt2^{+}. Mol. Microbiol. 42:29-36.

Pie de figuras

Figura 6.3.1 Síntesis de fitoquelatinas en cloroplastos intactos y puros de *Euglena gracilis*. Cloroplastos control (5 mg proteína/ml) se incubaron por 60 min con $CdCl_2$ 500 µM, la reacción se detuvo acidificando con PCA 3% final, la proteína se eliminó por centrifugación y las fitoquelatinas se cuantificaron por RP-HPLC. El compuesto X no fue identificado, pero su concentración no varió por la exposición a Cd^{2+}.

Figura 6.3.2 Velocidades iniciales de la PCS en extractos citosólicos de *Euglena gracilis*. Los cuadros representan la cantidad de PC_2 en cada punto de la curva, a los círculos se les restó la cantidad de PC_2 presente en el tiempo cero. 19.65 UA corresponden a 1 nmol de –SH. El inserto muestra el cromatograma con el cual se cuantificó la síntesis de PC_2.

Figura 6.3.3 Tioles solubles en ácido y contenido de Cd^{2+} en las fracciones de alto y bajo peso molecular del estroma.

Cloroplastos ZCd_{50} fueron solubilizados con Triton X-100 y fraccionados por ultrafiltración con un centricon (50 kDa). Ambas fracciones, de alto y bajo peso molecular, fueron analizadas por RP-HPLC y el Cd^{2+} se determinó por espectrofotometría de absorción atómica.

Tablas y Figuras

Tabla 6.3.1 Síntesis de fitoquelatinas en cloroplastos control y ZCd_{50} de *Euglena gracilis*.

	-Cd	+ Cd
	nmoles –SH / mg proteina	
Cloroplastos control		
Cys	1.2 ± 0.2	1.2 ± 0.3
GSH	0.13 ± 0.06	0.08 ± 0.03
γ-EC	0.44 ± 0.1	0.42 ± 0.2
X	0.03 ± 0.007	0.04 ± 0.02
PC_2	0.09 ± 0.01	0.18 ± 0.06*
PC_3	0.02 ± 0.01	0.07 ± 0.02*
PC_4	0.03 ± 0.006	0.036 ± 0.01
Cloroplastos ZCd_{50}		
Cys	2.4 ± 0.7	2.2 ± 0.5
GSH	5.3 ± 2.6	4.9 ± 2.1
γ-EC	4.7 ± 0.5	4.3 ± 0.7
X	0.4 ± 0.08	0.36 ± 0.15
PC_2	2.9 ± 0.09	2.7 ± 0.2
PC_3	0.8 ± 0.1	0.9 ± 0.3
PC_4	0.1 ± 0.05	0.11 ± 0.06

Promedio ± SD (n=3-4); * $P<0.05$ respecto al valor – Cd^{2+}. Los cloroplastos intactos (-Cd) se incubaron por 60 min con 0.5 mM $CdCl_2$ (+Cd) y posteriormente se determinó el contenido de tioles por RP-HPLC. X, compuesto no identificado.

Tabla 6.3.2 Localización intracelular de la PCS de *Euglena gracilis*

Fracción	PCS pmoles min^{-1} mg $proteína^{-1}$	GAPDH-$NADP^+$ nmoles min^{-1} mg^{-1}	GDHnmoles min^{-1} mg^{-1}	mg proteína / µg Chl
Extracto crudo	2.47 (20.9 µU/10^7 cels)	23 ± 8 (137 µU/10^7 cels)	5.2 ± 0.9	0.154 ± 0.01
Cloroplastos	1.81 (8.26 µU / 55 µgChl)*	15.4 ± 4 (70.3 µU / 55 µgChl)	0 ± 0	0.083 ± 0.009
Citosol	37.7 ± 6	634 ± 90	43.3 ± 9.7	23.7 ± 6

*La actividad de la PCS en cloroplastos corresponde al 39 % del total de acuerdo a los siguientes cálculos: la actividad de la PCS en cloroplastos se multiplicó por 0.083 para convertir las unidades a µg de Chl, posteriormente se multiplicó por 55 µg de Chl/10^7 cels para obtener la actividad proporcional a todos los cloroplastos de una célula. El mismo procedimiento se realizó para el sonicado, obteniendo el porcentaje de enzima asociada a cloroplastos. Excepto en la actividad de PCS en el extracto crudo (sonicado) y cloroplastos, todas las actividades son el promedio ± DE (n=3-6).

Tabla 6.3.3 Distribución de tioles en cloroplastos de *Euglena gracilis* expuestos a 50 µM de $CdCl_2$.

	Cys	γ-EC + GSH	X_1	PC_2	PC_3	PC_4	X_2	PC_5	X_3
					nmol -SH / fraction				
Total	7.3 ± 0.8	18 ± 3.6	0.06 ± 0.01	0.86 ± 0.2	0.25 ± 0.04	0.87 ± 0.1	0.45 ± 0.05	0.75 ± 0.2	0.26 ± 0.04
HMWC (>50 kDa)	3.6 ± 0.5 (49 %)	3.3 ± 0.6 (18 %)	0.05 ± 0.01 (83 %)	0.75 ± 0.2 (87 %)	0.23 ± 0.03 (92 %)	0.66 ± 0.06 (75 %)	0.45 ± 0.06 (73 %)	0.67 ± 0.2 (89 %)	0.24 ± 0.05 (92 %)
LMWC (<50 kDa)	3 ± 0.4	13.1 ± 1.2	N.D.	0.03 ± 0.01	N.D.	0.12 ± 0.05	0.08 ± 0.02	N.D.	N.D.

Cloroplastos ZCd_{50} purificados con Percoll (8-10 mg; 500 µl) solubilizados con 0.2% Tritón X-100 y centrifugados 20 min a 20 000 g. El sobrenadante se fraccionó con un Centricón (50 kDa). El concentrado se resuspendió en Tris 50 mM (pH 8.0) y se concentró por segunda vez. Ambas fracciones se analizaron por RP-HPLC. Los resultados son la media ± DE (n = 3). Los números entre paréntesis representan el porcentaje respecto al contenido en el estroma. La recuperación fue del 85-90%. El límite de detección fue 0.03 nmoles. X_1, péptido-tiol no identificado que permanece constante con y sin Cd^{2+}. X_2 y X_3, péptidos-tioles no identificados sintetizados en respuesta a Cd^{2+}, presumiblemente homo-fitoquelatinas. N.D., no detectado.

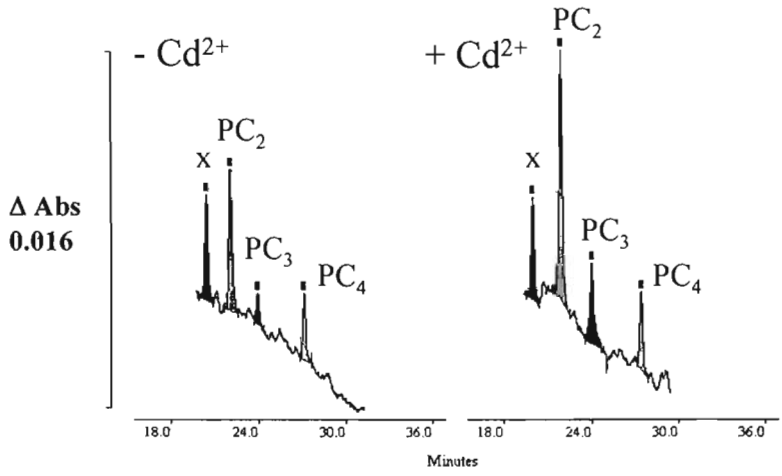


Figura 6.3.1

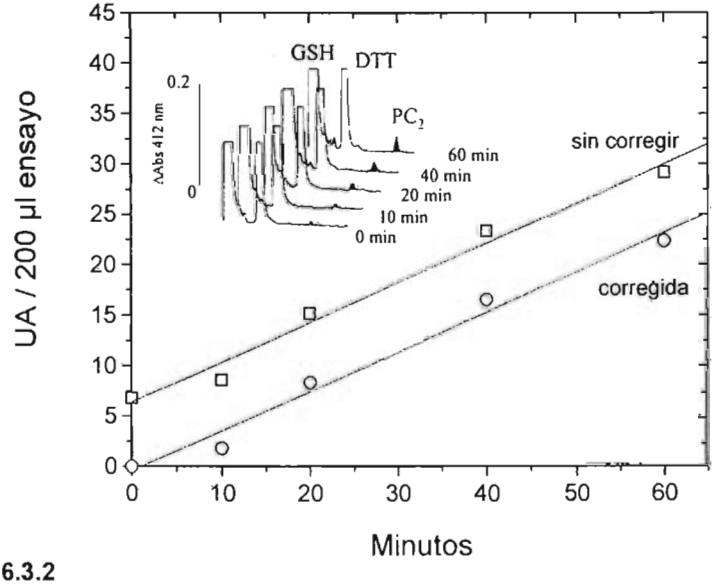


Figura 6.3.2

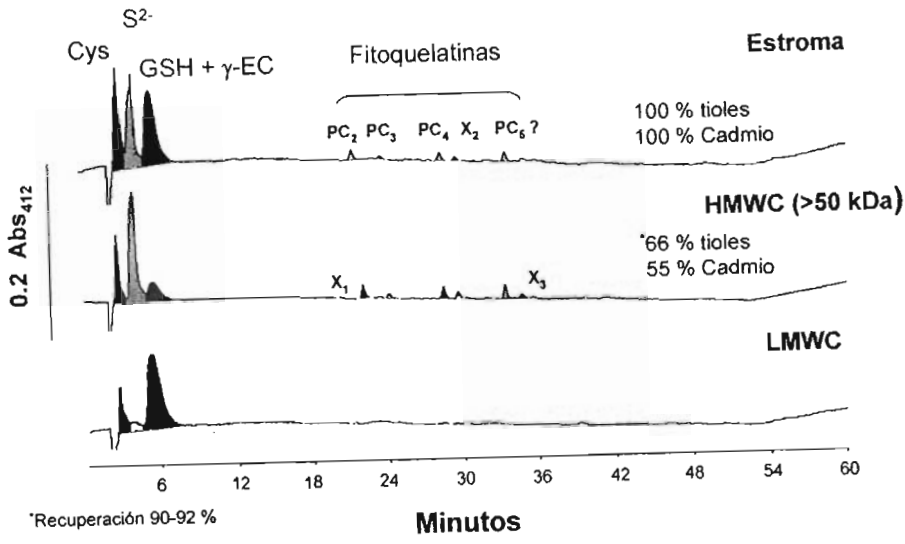

S2-
Cys
GSH + γ-EC
Fitoquelatinas
Estroma
PC2 PC3 PC4 X2 PC6 ?
100 % tioles
100 % Cadmio
HMWC (>50 kDa)
*66 % tioles
55 % Cadmio
X1
X3
LMWC
0.2 Abs412
6
12
18
24
30
36
42
48
54
60
*Recuperación 90-92 %
Minutos

Figura 6.3.3

6.4 Origen filogenético de la fitoquelatina sintetasa de *Euglena gracilis*

Las PCSs son enzimas que se encuentran en el citosol (Rea et al., 2004), por lo que el hallazgo de una PCS organelar tiene interesantes implicaciones filogenéticas.

El phylum Euglenozoa, que incluye a los órdenes Euglenida (*Euglena sp.*) y Kinetoplastida (*Trypanosoma sp.*), presenta una historia evolutiva muy característica. Aparentemente, el organismo eucarionte progenitor del phylum se separó del tronco común muy temprano en la evolución, justo después de haber adquirido las mitocondrias por endosimbiosis *primaria* (Gray et al., 1989, Hannaert et al., 2003). Posteriormente, y contrario al evento común de la adquisición del cloroplasto por una segunda endosimbiosis *primaria*, se llevó a cabo una endosimbiosis *secundaria*. Esto es, un organismo eucarionte (del phylum Euglenozoa) tuvo una endosimbiosis con otro organismo eucarionte (posiblemente un alga verde) del cual conservó principalmente el cloroplasto. Una evidencia de este proceso es la estructura atípica del cloroplasto de *E. gracilis*, el cual presenta 3 membranas además de las tilacoidales (Buetow, 1989). Durante este proceso se llevó a cabo una transferencia de genes del cloroplasto al genoma nuclear, un proceso bastante común tanto para mitocondrias como para cloroplastos (Gray et al., 1989).

Una diferencia entre los ordenes Euglenida y Kinetoplastida es que éste último perdió el cloroplasto pero conservó genes exclusivos de organismos fotosintéticos, además de desarrollar un organelo muy característico, derivado de los peroxisomas, denominado glicosoma, el cual es de vital importancia para el metabolismo de este organismo (Hannaert et al.,2003). La figura 6.4.1 muestra un árbol filogenético ilustrando estos eventos. Los organismos del orden Kinetoplastida son de relevancia clínica y, en el caso de *Trypanosoma brucei* y *T. cruzi*, sus respectivos genomas están prácticamente secuenciados (http://www.tigr.org). Un análisis de la base de datos de estos organismos

mostró que no hay genes homólogos de PCS lo cual sugiere que: (1) el phylum Euglenozoa NO tenía el gene de la PCS antes de la adquisición del cloroplasto, y por lo tanto adquirió éste del alga clorofita endosimbionte y (2) el orden Kinetoplastida no solo perdió el cloroplasto, sino algunos genes incluyendo el de la PCS. El hecho de haber encontrado actividad de PCS en el cloroplasto de *E. gracilis* apoya la idea de que esta enzima provino del alga endosimbionte.

A pesar de que esta hipótesis podría explicar el origen de la PCS de cloroplasto, quedaría incompleto el origen de la PCS en el citosol, el cual podría ser el resultado de una duplicación de genes (genes *ortólogos*) aunado a la pérdida del péptido de tránsito que dirige a la enzima al cloroplasto. En relación a ésto, en el alga verde *Chlamydomonas reinhardtii*, la cual tampoco tiene una vacuola tipo planta, el Cd^{2+} también se acumula en el cloroplasto (Nagel et al., 1996) y dentro de él se han encontrado compuestos similares a FQs (Nagel et al., 1996); sin embargo, no se conoce aún si dentro del cloroplasto de esta clorofita existe una PCS.

Además del origen filogenético, los resultados obtenidos sobre la caracterización funcional de la enzima nos permiten hacer un análisis función-estructura de la PCS de *E. gracilis*.

Las FQs fueron identificadas por primera vez en levaduras (*S. pombe*) y originalmente se les denominó *cadistinas* (Kondo et a., 1984). Hasta 1988 se identificaron compuestos homólogos a los cuales se les denominó FQs (Grill et al., 1988). Por una razón no muy clara, tal vez debido al mayor avance en el campo de las plantas que en el de las levaduras, el nombre que predominó en la literatura científica para estos compuestos fue FQs, el cual tiene un prefijo claramente erróneo si el término también se aplica a levaduras o a otros organismos.

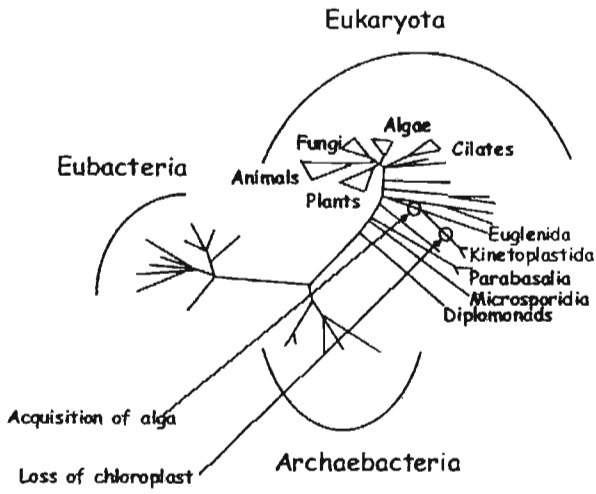


Figura 6.4.1 Árbol filogenético ilustrando la historia evolutiva del phylum Euglenozoa (tomado de Hannaert et al., 2003).

De cualquier manera, una vez determinada su estructura primaria [$(\gamma$-Glu-Cys$)_n$-Gly], donde n = 2-11, la cual guarda semejanza con el GSH, se sugirió que estas moléculas eran sintetizadas por una enzima a la cual se le denominó fitoquelatina sintetasa (revisado por Rea et al., 2004). La PCS de plantas (*Silene cucubalus*) se purificó y caracterizó en 1989 (Grill et al., 1989) y los genes, tanto de *S. pombe* como de plantas, se clonaron a partir de 1999 existiendo hasta la fecha al menos 5 secuencias reportadas (Rea et al., 2004). Una caracterización cinética mas profunda llevó a la conclusión de que la PCS es una dipeptidiltranspeptidasa cuya reacción se muestra en la figura 6.4.2A, y el ciclo catalítico propuesto en la figura 6.4.2B; nótese que durante la síntesis de una FQ se libera una Gly y por lo tanto se genera un intermediario des-Gly, que en el caso de que el

donador del grupo γ-EC fuera GSH, sería γ-EC y en el caso de que el donador fuera otra FQ el intermediario sería una des-Gly-FQ.

La era post-genómica y la biología molecular han hecho posible dos hallazgos importantísimos para el origen y función de las PCS: (1) se clonó y se determinó que el gusano *Caenorhabditis elegans*, un nematodo marino, posee una PCS funcional y (2) la cianobacteria *Nostoc sp.* contiene un gen homólogo, pero incompleto (correspondiente al extremo amino terminal de las PCS "canónicas"), el cual se ha sugerido como el ancestro de la enzima y que proviene, por cierto, de un organismo fotosintético relacionado con el origen del cloroplasto.

La figura 6.4.3 muestra un esquema que compara diferentes secuencias de PCSs haciendo especial énfasis en los aminoácidos esenciales para la catálisis y las diferencias entre secuencias a nivel del carboxilo terminal. Varios experimentos nos permiten hacer inferencias respecto a la estructura-función de la PCS de *Euglena*. Los fragmentos de la secuencia AtPCS1 denominados PCS_Nt1 y PCS_Nt2 son fragmentos proteolíticos de la secuencia original AtPCS1 con un carboxilo terminal más corto (Ruotolo et al., 2004). Estos fragmentos son capaces de sintetizar FQs pero solo de cadena corta (n=2-3) e incapaces de sintetizar FQs de cadena larga (> 4). Por otro lado, la proteína codificada por el gen Alr0975 de *Nostoc sp.*, cuyo carboxilo terminal esta ausente, preferentemente hidroliza GSH a γ-EC + Gly mientras que la síntesis neta de FQs es muy ineficiente

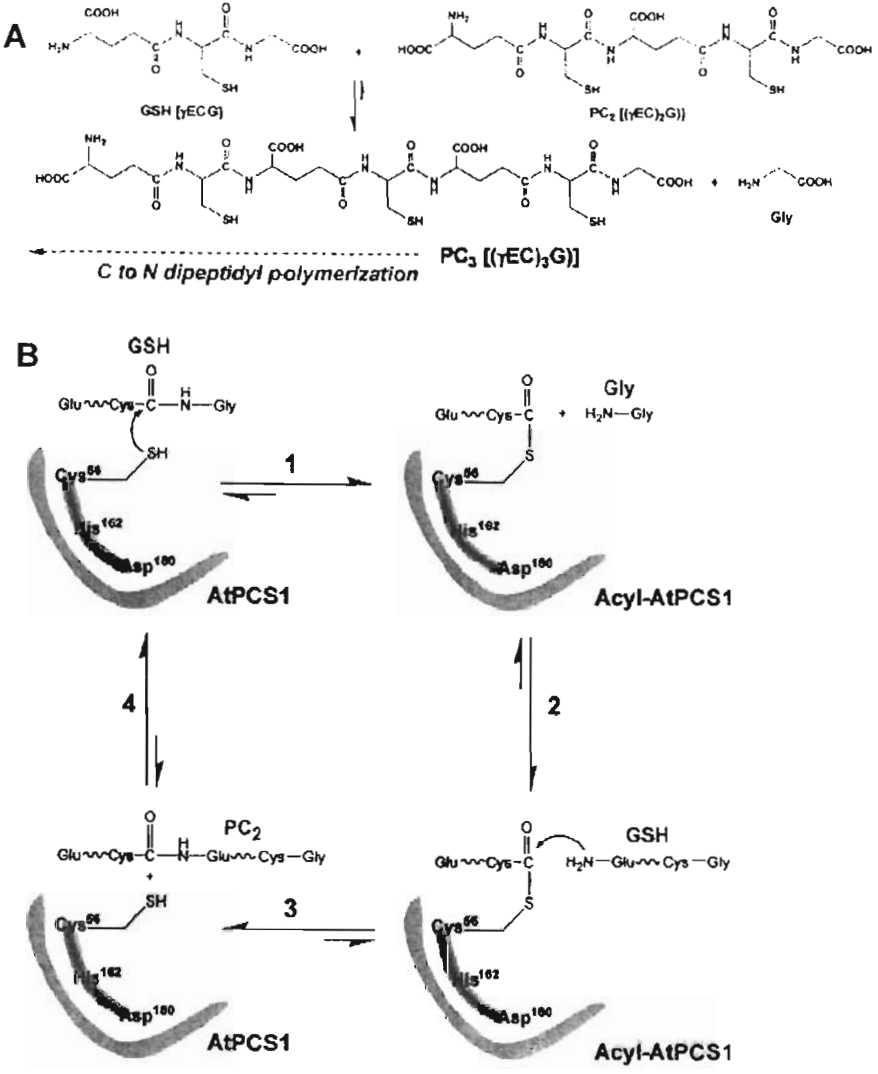


Fig 6.4.2 (**A**) Reacción de la PCS y (**B**) ciclo catalítico propuesto. (tomado de Rea et al.,2004).

Estos resultados sugieren que el segmento amino terminal de la PCS está relacionado con la catálisis e hidrólisis, mientras que el segmento del carboxilo terminal está relacionado con la longitud de las FQs sintetizadas (Ruotolo et al., 2004). Hay que mencionar que la síntesis de FQs de cadena larga en plantas puede llevarse a cabo en horas, y su acumulación depende de los días de exposición a Cd^{2+}.

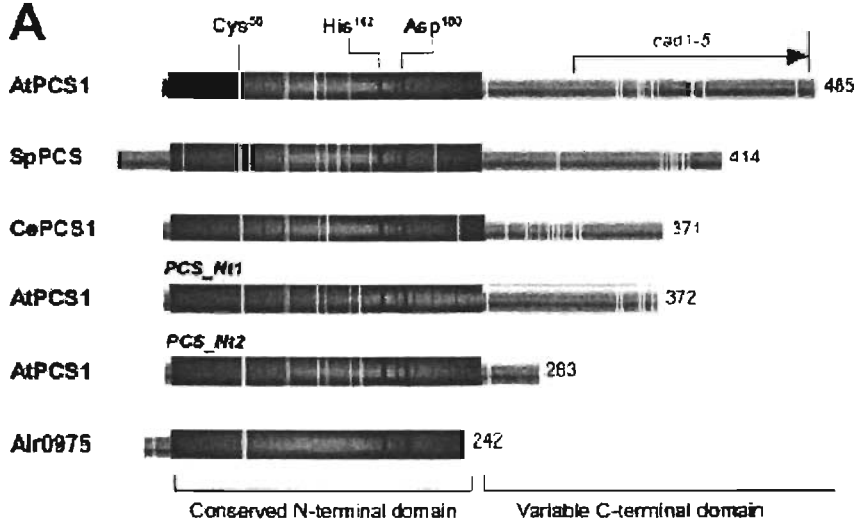


Figura 6.4.3 Estructura de diferentes fitoquelatina sintetasas con énfasis en los aminoácidos esenciales del sitio activo y el dominio carboxilo altamente variable entre secuencias (tomado de Rea et al.,2004)

La figura 6.4.4 muestra la síntesis de FQs en *E. gracilis* después de ocho días de exposición a diferentes concentraciones de Cd^{2+}. Es notable que aún después de 8 días de exposición a Cd^{2+} las FQs de mayor abundancia sean de cadena corta (2-3). Por otro

lado, utilizando extractos de *E. gracilis*, hemos hecho experimentos en condiciones de síntesis de FQs para detectar hidrólisis de GSH y en ningún caso hemos detectado formación de γ-EC. Estos resultados sugieren que la PCS de *Euglena* tiene mayoritariamente actividad de PCS, por lo que debe tener un carboxilo terminal similar a plantas, pero lo suficientemente distinto como para sintetizar preferentemente FQs de cadena corta.

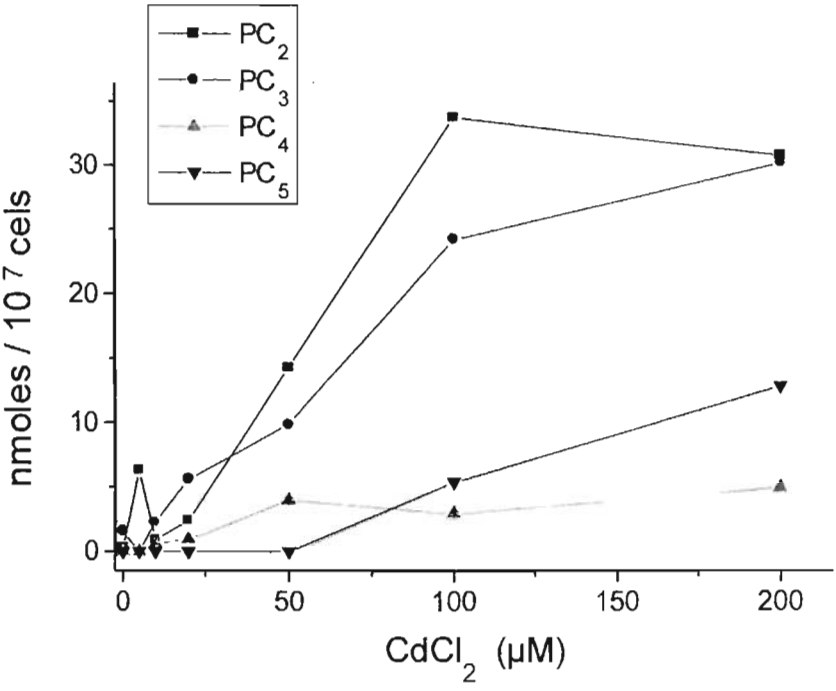


Figura 6.4.4 Síntesis de fitoquelatinas en *Euglena gracilis* después de 8 días de exposición a diferentes concentraciones de Cd^{2+}.

6.5 Caracterización de los complejos tiol-Cd^{2+}-sulfuro (HMWC) en *Euglena gracilis* *

Como se revisó en los capítulos 2 y 6, un proceso indispensable para la inactivación del Cd^{2+} es la formación de complejos de alto peso molecular formados por tioles, Cd^{2+} y sulfuro (HMWC), alrededor de un núcleo de sulfuro de cadmio (CdS; Figura 6.5.1). En plantas y algunas levaduras como *S. pombe* y *C. glabrata,* estos complejos se localizan principalmente en la vacuola (Barbas et al., 1992; Speiser et al., 1992; Vande y Ow, 2000). Su formación aparentemente es no-enzimática y pueden formarse *in vitro* por la simple combinación de sus componentes (Cd^{2+}, tioles y sulfuro); sin embargo, *in vivo* son necesarias dos enzimas relacionadas con la síntesis de purinas: la adenilosuccinato sintetasa y la succinoaminoimidazol carboximida sintetasa, las cuales pueden utilizar un análogo del aspartato, sulfinato de cisteína, para transferir el sulfuro a los complejos (Juang et al., 1993). Otra característica importante de estos complejos es que, en plantas y *S. pombe*, los HMWC están formados únicamente por Cd^{2+}, sulfuro y FQs, mientras que en *C. glabrata* otros tioles (monotioles), como γ-EC y GSH, pueden incorporarse en los complejos. Se ha demostrado que la composición de tioles esta relacionada con la estabilidad de los HMWC. Los complejos que incluyen monotioles resultan ser mucho más inestables fuera de la célula que los complejos formados sin monotioles (Dameron et al., 1989).

Ya demostramos que en el cloroplasto de *Euglena* los tioles y el Cd^{2+} se encuentran en la fracción de alto peso molecular del estroma; sin embargo, su estructura y composición no se ha determinado.

*** En este trabajo participaron: Mendoza-Cózatl D, Mendoza-Hernández G y Moreno-Sánchez R.**

Además, dos observaciones hacen suponer que estos complejos **NO** son idénticos a los reportados para plantas y levaduras: (1) su localización intracelular, pues las condiciones del cloroplasto son muy diferentes a la vacuola respecto al pH y disponibilidad de tioles (al contrario del cloroplasto, en la vacuola prácticamente no hay GSH) y (2) la cuantificación de tioles solubles en ácido en *Euglena*, tanto en células como en cloroplastos, ha mostrado que respecto al total de tioles, la mayor parte son monotioles (Cys, γ-EC y GSH) y solo una fracción menor corresponde a FQs. Por lo tanto, sí la formación de los HMWC es no-enzimática, esperaríamos que la composición de los HMWC de *Euglena* fuera más parecida a *C. glabrata* que a plantas y *S. pombe*.

En el siguiente trabajo, purificamos los HMWC de *Euglena* para determinar sus propiedades fisicoquímicas tales como peso molecular, propiedades ópticas, composición de péptidos y aminoácidos así como la estequiometría tiol-Cd^{2+} -sulfuro, la cual será definitiva para establecer si estos complejos están relacionados con la inactivación del Cd^{2+} en *E. gracilis*.

Debido al bajo rendimiento en la obtención de cloroplastos puros e intactos, se decidió como una primera aproximación, que la purificación de los HMWC de *Euglena* se realizara a partir de células enteras. Puesto que la mayor parte del Cd^{2+} y FQs se encuentran en el interior de este organelo, la información obtenida de células enteras sería un reflejo de los HMWC formados dentro del cloroplasto.

Material y Métodos

Purificación de los HMWC de Euglena

Los HMWC se purificaron de acuerdo a la técnica reportada por Grill et al. (1991) partiendo de citosol obtenido de células cultivadas por 8 días con 50 μM $CdCl_2$. El citosol

se obtuvo por sonicación exhaustiva de las células (hasta que el 90 % de las células fueran rotas) y centrifugación a 225 000 x *g* por 60 min. Todos los buffers usados durante la purificación fueron previamente saturados con N_2. El citosol se cargó en una columna de DEAE (1.5 x 25 cm; DEAE-Biogel) equilibrada con Tris 10 mM pH 8.0 (Buffer A) a un flujo de 200 ml/h. Una vez cargada la muestra, la columna se lavó con 50 ml de Buffer A. Los compuestos unidos se eluyeron en un solo paso con NaCl 0.5 M en el buffer A. Toda la proteína fue colectada y llevada a 80% de saturación con $(NH_4)_2SO_4$ (0.6 g / ml de eluado). La proteína precipitada se removió por centrifugación a 9000 x *g* por 10 min y el sobrenadante se diluyó 1:1 con buffer A y posteriormente se concentró con un Amicon (10 kDa) hasta 1 ml. El concentrado final se sometió a una cromatografía de filtración en gel (Sephacryl S-200, 1.7 x 56 cm) a un flujo de 60 ml/h, colectando fracciones de 4 ml. Las fracciones con absorbencia (280 nm) se colectaron y liofilizaron para posteriormente ser analizadas por RP-HPLC y determinar el contenido de tioles, Cd^{2+} y sulfuro de la misma manera que lo reportado previamente (Mendoza-Cózatl y Moreno-Sánchez, 2005). El rendimiento a partir de 2.8 x 10^8 células (1.7 g de peso húmedo) fue de 7 mg de HMWC.

Caracterización de los HMWC de Euglena gracilis

La composición de aminoácidos de los HMWC (1 mg) se llevó a cabo después de mezclarlos con HCl 1 N, e hidrolizarlos a 100°C durante 24 h y posteriormente derivatizarlos con fenilisotiocianato. La separación y cuantificación se llevó a cabo por RP-HPLC. La Cys se modificó con poliacrilamida o se cuantificó con 1 mM de DTNB en un buffer de Tris 50 mM, 10 mM EGTA pH 8.0 usando un ε_{TNB} 13,600 M^{-1} cm^{-1} a 412nm.

Marcaje de HMWC con $^{109}Cd^{2+}$

HMWC (30 µg) fueron incubados con 75 µCi de $^{109}CdCl_2$ durante 30 min a 4 °C en un buffer con 50 mM Tris pH 8.0, previamente saturado con N_2, en un volumen final de 30 µl. Los HMWC marcados se analizaron en un gel de poliacrilamida (10%) desnaturalizante (0.1 % SDS) en condiciones no reductoras de acuerdo al método de Laemmli (1970).

Medición de proteína

Debido a que las FQs no dan señal por los métodos tradicionales (Bradford o Biuret), la proteína se cuantificó por el método de Murphy y Kies (1960) midiendo la absorbencia del enlace peptídico (Abs_{215nm}-Abs_{225nm}) y usando BSA como estándar.

Resultados y Discusión

La figura 6.5.2 muestra el peso molecular nativo de los HMWC de *E. gracilis* (148 ± 25 kDa; media ± DE, n =3) los cuales se purificaron como un compuesto homogéneo a juzgar por el perfil cromatográfico (Fig. 6.5.2, inserto), sugiriendo que en *Euglena* solo hay una especie molecular de HMWC. El análisis espectrofotométrico de los HMWC mostró 2 máximos de absorción, uno a 220 nm (enlace peptídico) y otro a 254 nm (enlace tiol-Cd; Fig. 6.5.3). La acidificación de los HMWC con HCl (10% concentración final), indujo una reducción en la absorbencia a 254 nm pero no la de 220 nm, lo cual es consistente con la idea de que el Cd^{2+} está unido, al menos parcialmente, a través de grupos tioles. La acidificación induce la protonación del grupo tiol desplazando al Cd^{2+}, reduciendo así la absorbancia a 254 nm (Dameron et al., 1989; Barbas et al., 1992). No se observaron hombros a 280 nm, lo que indica poca o nula contaminación por proteínas (con aminoácidos aromáticos). Un análisis adicional de los HMWC por SDS-PAGE (10%, no-reductor) teñido con azul de Coomassie, tampoco reveló contaminación por proteínas (Fig.

6.5.4A); sin embargo, cuando se marcaron HMWC con $^{109}Cd^{2+}$, estos revelaron un compuesto de más de 100 kDa (Fig. 6.5.4B). Si bien el peso molecular no corresponde con aquel determinado por filtración en gel (148 kDa), desconocemos la estabilidad de los HMWC fuera de la célula y además, desconocemos su comportamiento en un campo eléctrico, por lo que el menor peso puede corresponder a HMWC parcialmente disociados.

El análisis por RP-HPLC mostró que los HMWC de *Euglena* están formados principalmente por monotioles (Cys + γ-EC + GSH = 74.8%) y solo un 15 % por FQs (Fig. 6.5.5). Los HMWC de *Euglena* contienen una elevada cantidad de S^{2-}, la cual junto con el Cd^{2+} determinado en los complejos da una relación S^{2-}/Cd^{2+} de 1.87 (Tabla 6.5.1). Esta relación es semejante a la reportada en plantas y *S. pombe* (Speiser et al., 1992). Por otro lado, la relación -SH + S^{2-} /Cd^{2+} fue de 3.41 (Tabla 6.5.1), un valor superior al encontrado en algunas plantas (-SH + S^{2-} /Cd^{2+} =1.18 para maíz) y similar a lo reportado para *S. pombe* (-SH + S^{2-} /Cd^{2+} = 4) (Speiser et al., 1992). Los resultados sugieren que, al igual que en plantas y algunas levaduras, los HMWC de *Euglena* están relacionados con la inactivación-acumulación de Cd^{2+}.

Por otro lado, la composición de aminoácidos de los HMWC reveló que el 72.4 % de los HMWC están formados por Glu, Cys, Gly, Ser y Ala, los cuales pueden pertenecer a γ-EC, GSH, homo-glutatión y FQs (Tabla 6.5.2). Sorprendentemente, el Asp constituyó un 19 % de los aminoácidos totales, un porcentaje muy superior al contenido promedio de este aminoácido en una proteína (5%; Lehninger et al., 2000) para sospechar de una posible contaminación. Al momento desconocemos el origen de este aminoácido y/o si forma parte de los complejos como aminoácido libre.

Los HMWC de plantas y *S. pombe* tienen un peso molecular de entre 10-30 kDa, se encuentran principalmente en la vacuola y están formados exclusivamente por Cd^{2+},

sulfuro y FQs (Speiser et al., 1992). Los HMWC de *C. glabrata* pueden contener, además de FQs, monotioles tales como γ-EC y GSH (Dameron et al., 1989). Aparentemente, la incorporación de estos monotioles disminuye la estabilidad a los complejos (Dameron et al., 1989). Al igual que los complejos de *C. glabrata*, los HMWC de *Euglena* contienen cantidades significativas de monotioles (74.8%) y al igual que en esta levadura, los HMWC de *Euglena* son inestables tal y como lo muestra la electroforesis no reductora (Fig. 6.5.4 A-B).

Por otro lado, en *S. pombe* y *C. glabrata* se ha demostrado que los HMWC se forman alrededor de un núcleo de CdS, y dependiendo del tamaño del cristal, los HMWC pueden mostrar transiciones electrónicas en longitudes de onda cercanos al ultravioleta (300-330 nm, Barbas et al., 1992). Un núcleo de CdS de 20 Å de diámetro, formado por aproximadamente 85 moléculas de CdS, presenta una transición a 316 nm (Barbas et al., 1992). Debido a que los HMWC de *Euglena* no presentan ninguna transición en longitudes mayores de 300 nm, podemos concluir que el núcleo de estos complejos es menor a 20 Å (Fig. 6.5.3)

Nosotros hemos demostrado que en *Euglena*, la mayor parte de las FQs se encuentran en el cloroplasto y que dentro de este organelo, más del 80% se encuentran como complejos de alto peso molecular (Mendoza-Cózatl et al., 2002; ver capitulos 2 y 6.1). Los HMWC caracterizados en este trabajo se purificaron a partir de células enteras, si bien no podemos decir que la totalidad de los complejos estén en el cloroplasto, si podemos sugerir que la mayor parte de ellos esta en este organelo y, por otro lado, debido a que los HMWC se obtuvieron como un solo compuesto homogéneo (Fig. 6.5.2, inserto), podemos decir que en *Euglena* hay un solo tipo de HMWC.

Independientemente de si los HMWC de *Euglena* se forman en el citosol o en el cloroplasto, cabe señalar que ambos compartimentos tienen características que difieren significativamente de las condiciones en la vacuola, principalmente en valores de pH y disponibilidad de monotioles (en la vacuola no hay cantidades significativas de GSH). Además de la diferencia en el tamaño del núcleo de CdS, los HMWC de *Euglena* también difieren significativamente de los encontrados en plantas y levaduras respecto al peso molecular, composición de péptidos y aminoácidos. Estas diferencias pueden deberse a la disponibilidad de tioles y las condiciones del compartimento celular donde se forman.

Referencias

Barbas J, Santhanagopalan V, Blaszczynski M, Ellis WR, Winge DR (1992) Conversion in the peptides coating cadmium:sulfide crystallites in *Candida glabrata*. 48:95-105.

Dameron CT, Smith BR, Winge DR (1989) Glutathione-coated cadmium-sulfide crystallites in Candida glabrata. 264:17355-17360.

Grill E, Winnacker EL, Zenk MH (1991) Phytochelatins. Methods Enzymol. 205:333-341.

Juang RH, McCue KF, Ow DW (1993) Two purine biosynthetic enzymes required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. Arch. Biochem. Biophys. 304: 392–401.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lehninger A.L., Nelson D.L., Cox M.M. (2000) Principles of biochemistry. 3rd edn., Worth Publishers, Inc. New York 1152 pp. ISBN 1-57259-153-6

Mendoza-Cózatl D, Devars S, Loza-Tavera H, Moreno-Sánchez R (2002) Cadmium accumulation in the chloroplast of *Euglena gracilis*. Physiol. Plant. 115:276-283.

Mendoza-Cózatl D, Moreno-Sánchez R. (2005) Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*. Biochim. Biophys. Acta 1706: 88-97.

Murphy JB, Kies MW (1960) Note on spectrophotometric determination of proteins in dilute solutions. Biochim Biophys Acta 45:382–384.

Speiser DM, Abrahamson SL, Bañuelos G, Ow DW (1992) *Brassica juncea* produces a phytochelatin-cadmium-sulfide complex. Plant Physiol. 99:817-821.

Vande WJG, Ow DW (2001) Accumulation of metal-binding peptides in fission yeast requires hmt2^{+}. Mol. Microbiol. 42:29-36.

Pies de figura

Figura 6.5.1 Estructura propuesta para los HMWC formados por Cd^{2+}, FQs y sulfuro de plantas. Este tipo de estructura se formaría alrededor de un núcleo de CdS (Tomado de Pickering et al., 1999).

Figura 6.5.2 Determinación del peso molecular de los HMWC de *E. gracilis* utilizando una columna de exclusión molecular Sephacryl S-200 (1.7 x 56 cm). Los estándares utilizados se muestran en círculos abiertos y el número se refiere al peso molecular. El coeficiente de distribución (K_{av}) se determinó de acuerdo a la fórmula $K_{av} = Ve\text{-}Vo/Vt\text{-}Vo$, donde *Ve* es el volumen de elución del compuesto de interés, *Vo* es el volumen vacío (60 ml determinado con Azul Dextran) y *Vt* el volumen total (127 ml).

Figura 6.5.3 Propiedades ópticas de los HMWC de *Euglena gracilis*.

Figura 6.5.4 SDS-PAGE no-reductor de los complejos de alto peso molecular de *E. gracilis*.

(**A**) 20 µg de proteína de los HMWC se analizaron en un gel de poliacrilamida (10%) sin β-mercaptoetanol, se incluyeron 20 µg de proteína de citosol como control positivo. (**B**) Radioblott de los HMWC de *Euglena*, 20 µg de proteína de los HMWC se incubaron con 75 µCi de $^{109}CdCl_2$ y se separaron de la misma manera que en el inciso A.

Figura 6.5.5 Perfil de RP-HPLC de los compuestos tioles de los complejos de alto peso molecular purificados a partir de células cultivadas con $CdCl_2$ 50 µM durante 8 días.

Tablas y Figuras

Tabla 6.5.1 Contenido de tioles, sulfuro y Cd^{2+} de los complejos de alto peso molecular purificados a partir de células cultivadas con 50 µM de $CdCl_2$

Cys	γ-EC + GSH	PCs	Cd^{2+}	S^{2-}	-SH	-SH + S^{2-} / Cd^{2+}
	(%)		nmoles / mg proteina HMWC			
6.4	68.4	25.1	67.7	126.7	105.7	3.41

Promedio de dos determinaciones

Tabla 6.5.2 Composición de aminoácidos de los complejos tiol-Cd^{2+}-sulfuro de *E. gracilis* (1 mg proteína) purificados a partir de células cultivadas con 50 µM de $CdCl_2$.

Aminoácido	Contenido Intracelular (nmoles / mg HMWC)	Contenido Porcentual Observado
Glu	21.1	27.9
Cys	25.09* (0.63)	33.2
Gly	5.4	7.1
Ser	0.62	0.82
Ala	2.56	3.39
Asp	14.4	19
Thr	1.4	1.8
Tyr	1.36	1.8
Pro	1.1	1.4
Val	0.95	1.3
Phe	0.8	1.5
Ile	0.68	0.9

* Determinado con DTNB, el número entre paréntesis fue el encontrado por derivatización con fenilisotiocianato. La determinación de aminoácidos solo se ha llevado a cabo una vez.

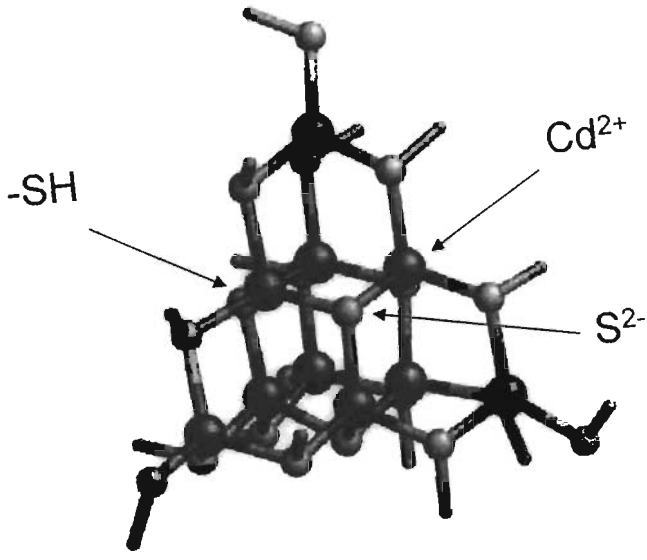


Figura 6.5.1

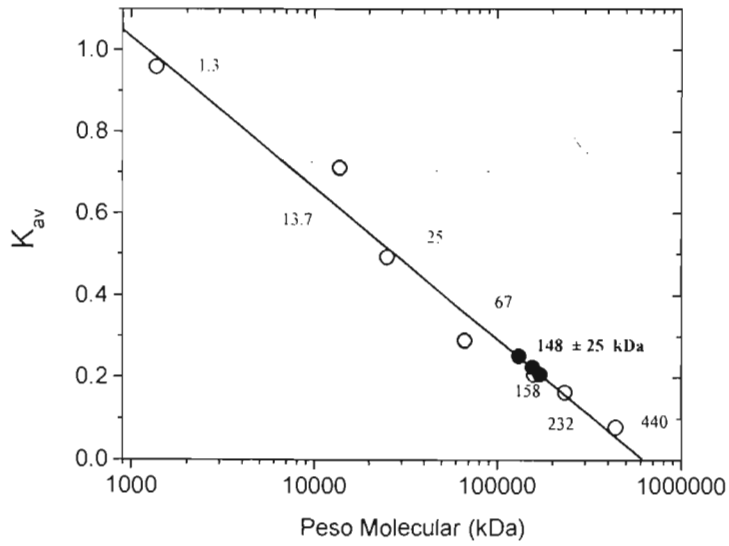


Figura 6.5.2

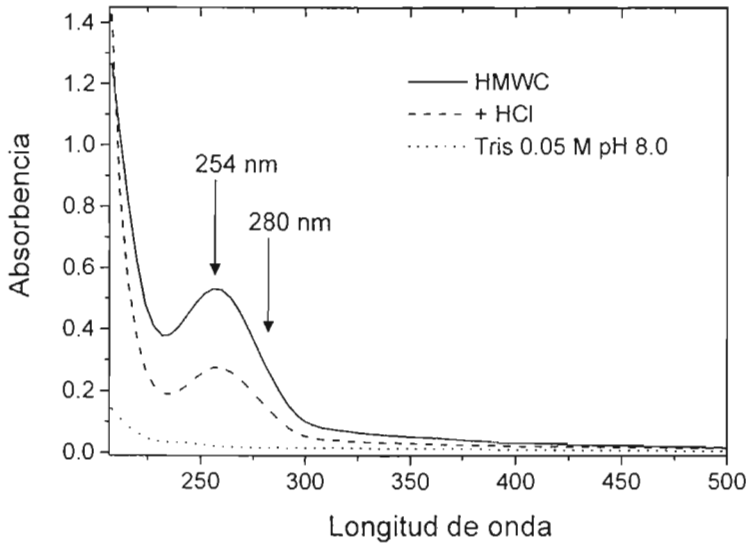
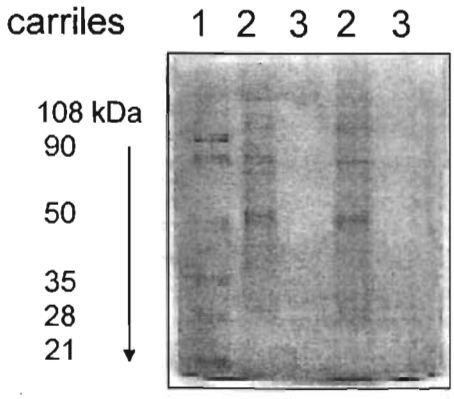


Figura 6.5.3

A



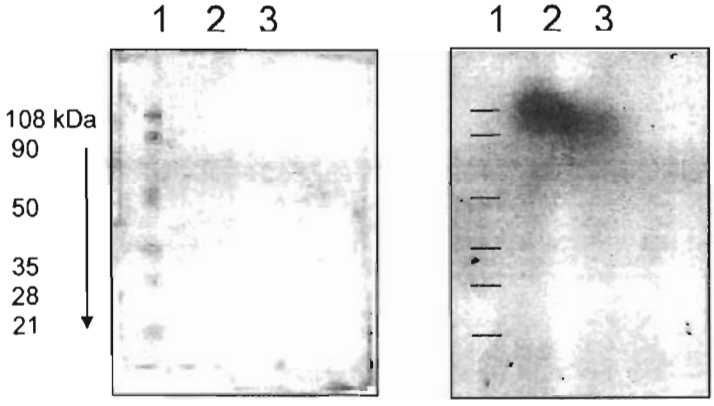
carriles

1.- Std. PM

2.- 20µg Citosol

3.- 20µg HMWC

B



carriles

1.- Std. PM

2.- 20µg HMWC + $^{109}Cd^{2+}$

3.- 20µg HMWC

Figura 6.5.4

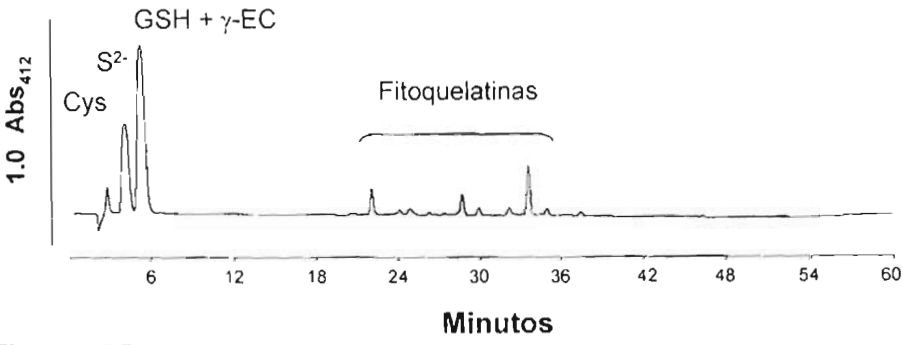

GSH + γ-EC
S2-
Cys
Fitoquelatinas
1.0 Abs412
6
12
18
24
30
36
42
48
54
60
Minutos

Figura 6.5.5

Capitulo 7 Capacidad de biorremoción de cadmio, zinc y plomo por *Euglena gracilis*

Hasta este punto, la tesis presentada se ha centrado en el mecanismo bioquímico por el cual *E. gracilis* resiste y acumula cantidades significativas de Cd^{2+}. Si bien se han cumplido los objetivos planteados al inicio de la tesis, queda incompleta la información acerca de qué tan factible es el uso de este organismo en procesos de biorremoción de metales de cuerpos de agua. Este es un problema común cuando se trata de hacer una conexión entre ciencia *básica* y *aplicada*. No necesariamente debe haber una conexión entre ambas, pero sería ideal que el avance en ciencia *básica* tuviera un uso inmediato en la ciencia *aplicada*. El siguiente trabajo hace precisamente esta conexión. A pesar de no estar incluido en los objetivos planteados al inicio de la tesis, el trabajo establece una relación entre el mecanismo de resistencia-acumulación de Cd^{2+} en *E. gracilis* y la viabilidad de este organismo para ser usado en procesos de remoción de metales de sistemas acuosos. Además, no solo determina la capacidad de *Euglena* para remover Cd^{2+}, adicionalmente analiza cómo otros metales usualmente presentes en aguas residuales, como zinc y plomo, pueden afectar esta capacidad de remoción. Por estas razones, procedimos a desarrollar el siguiente trabajo como parte del trabajo de tesis.

p 121-151 Archives of Environmental Contamination and Toxicology

(enviado para su evaluación)

Simultaneous Cd^{2+}, Zn^{2+} and Pb^{2+} uptake and accumulation by photosynthetic *Euglena gracilis*

David G. Mendoza-Cózatl*, Edna Rangel-González and Rafael Moreno-Sánchez

Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano 1, Sección XVI Tlalpan, 14080 México D.F. MÉXICO.

Running title: Removal of cadmium, zinc and lead by *Euglena gracilis*

Key Words: bioremediation, thiol-containing compounds, glutathione, phytochelatins, compartmentation.

***Corresponding author**

David G. Mendoza-Cózatl, Ph. D.
Departamento de Bioquímica
Instituto Nacional de Cardiología
Juan Badiano # 1, Col. Sección XVI, Tlalpan 14080
México, DF. MÉXICO.
Phone: (52)55-5573-2911 Ext. 1422
Fax: (52)55-5573-0926
Email: dgmcozatl@yahoo.com.mx

Abstract

The ability of *Euglena gracilis* to simultaneously remove and accumulate Zn^{2+}, Cd^{2+} and Pb^{2+} from the culture media was evaluated. *E. gracilis* was able to remove up to 80% of the Cd^{2+} present in the medium when cultured with 20 or 50 μM $CdCl_2$. Higher external Cd^{2+} concentrations increased Cd^{2+} accumulation *per* cell but decreased the cell density, thus diminishing the capacity of the cell culture to remove Cd^{2+}. *E. gracilis* removed 60% of the Zn^{2+} present in the medium when cultured with 5 to 50 μM $ZnSO_4$. Zn^{2+} did not affect the Cd^{2+} removal capacity. *E. gracilis* was much less efficient in removing Pb^{2+} (<20%) when cultured with 100 or 200 μM $Pb(NO_3)_2$. Moreover, Pb^{2+} decreased the efficiency to remove Cd^{2+}; Pb^{2+} did not affect Zn^{2+} removal. Cd^{2+} induced a generalized increase in the cellular thiol-compounds, including phytochelatins, and Pb^{2+} had an additive effect only at 200 μM. Zn^{2+} did not stimulate phytochelatin synthesis. Cd^{2+} and Pb^{2+} co-located in the cytosol at the same high-molecular weight fraction. As Pb^{2+} is a weak phytochelatin inducer, competition between Pb^{2+} and Cd^{2+} for transportation across the plasma membrane and binding to phytochelatins and other thiol-compounds is proposed to explain the detrimental effects of Pb^{2+} on the Cd^{2+} removal capacity of *E. gracilis*.

Introduction

Industrial activity and the inappropriate disposal of residues have turned heavy metal pollution in a serious environmental problem. Removal of heavy metals from polluted waters by the use of plants and microorganisms, a process called "bioremediation", is an expanding technology with several advantages over physical remediation methods (Salt et al., 1995; Dhankher et a., 2002). Thus, several works have studied the ability of bacteria, plants, yeast, and micro- and macro-algae to tolerate, bind and accumulate heavy metals (Trevors et al., 1996; Salt et al., 1995; Nedelkoska and Doran, 2000; Hamdy, 2000; Dhankher et a., 2002).

Euglena gracilis is a photosynthetic, free-living, unicellular protist with high resistance to heavy metals such as Hg^{2+}, Cd^{2+}, Cr^{6+} and Pb^{2+} and several groups have characterized some of the biochemical mechanisms involved in this process (Bariaud et al., 1985; Coppellotti, 1989; Navarro et al., 1997; Devars et al., 1998; Devars et al., 2000; Cervantes et al., 2001; Einicker-Lamas et al., 2003). For instance, a decreased Cd^{2+} uptake accounts for the differential Cd^{2+} sensitivity in resistant and sensitive strains of photosynthetic *E. gracilis* (Bariaud et al., 1985; Devars et al., 1998). Also, enhanced content of thiol-compounds (Cys, glutathione, and phytochelatins) together with active intracellular compartmentation into chloroplasts and mitochondria has been described for Cd^{2+} resistance in *E. gracilis* (Mendoza-Cózatl et al., 2002; Avilés et al., 2003). Volatilization and increased levels of glutathione were also described for Hg^{2+} resistance in *E. gracilis* (Devars et al., 2000).

In addition to high Cd^{2+} resistance (IC_{50} = 100 μM for photosynthetic strain Z; Devars et a., 1998), high Cd^{2+} accumulation capacity has also been reported for *E. gracilis*: heterotrophic cells exposed to 200 μM $CdCl_2$ for 96 h accumulated 11.2 g Cd^{2+}/ Kg dry weight (1.1 %) (Avilés et al., 2005). Therefore, *E. gracilis* may be a suitable model for bioremediation of heavy metal-polluted water bodies (Devars et al., 1998; Mendoza-Cózatl et al., 2002). However, few works, if any, have determined the ability of *E. gracilis* to remove heavy metals from aqueous solutions, either synthetic or natural wetlands. Furthermore, most of the experiments related to heavy metal removal using free-living organisms have been performed with only one heavy metal (Kaplan et al., 1995; Carr et al., 1998; Matsunaga et al., 1999).

Although necessary as a first step to characterize the utility of a given organism for bioremediation of one particular heavy metal, it should be recall that polluted waters usually contain a mixture of heavy metals, which may compete with each other for binding and transportation into the cell (Guerinot, 2000; Williams et al., 2000). In addition, cells may use different resistance mechanisms, depending on the heavy metal and its concentration. Thus, the presence of a given heavy metal may be detrimental for the detoxification of another one, affecting the ability of an organism to accumulate both heavy metals, simultaneously. On the contrary, exposure to a given heavy metal may stimulate the response against oxidative stress or exposure to another toxic heavy metal, improving their overall performance (*i.e.*, growth) under unfavorable conditions (Devars et al., 1998; Tsuji et al., 2002).

In the present work the ability of *E. gracilis* to remove Cd^{2+}, Zn^{2+} and Pb^{2+}, separately or in combination from the culture medium was assessed. As the content

of thiol-compounds is directly related to Cd^{2+} resistance and accumulation capacity in *E. gracilis* (Coppellotti, 1989; Devars et al., 1998; Mendoza-Cózatl et al., 2002; Avilés et al., 2003), the different acid-soluble thiol-compounds (cysteine, γ-glutamylcysteine, glutathione, phytochelatins) were also analyzed to determine whether Zn^{2+} and Pb^{2+} have an additive effect on the glutathione-phytochelatin synthesis pathway or they are detoxified by different mechanisms.

Material and Methods

Cell culture and growth conditions

Axenic cultures of *Euglena gracilis* Klebs (strain Z) were grown under photoheterotrophic conditions with a light/dark cycle of 12 h, as described by Devars et al. (1998). Cultures were initiated with a cell inoculum of 0.4×10^6 cells/ml. $CdCl_2$, $ZnSO_4$ and $Pb(NO_3)_2$ were added from sterile stock solutions. When Zn^{2+} concentration was modified, sulfate was maintained constant in the culture (2.7 mM) by adding H_2SO_4 before adjusting the final pH value of the medium (pH 3.5). Growth was determined by counting cells from culture aliquots in a Neubauer chamber, after appropriate dilution and immobilization with HCl. Cell viability, measured by the trypan blue method (described in Devars et al., 1998), was always higher than 90%. Cells were harvested at the eight day of culture (early stationary phase for control cells) by centrifugation at 1 000 x *g* for 10 min (4°C) and washed twice with 50 mM Tris-HCl, pH 8.0. The pellet was finally re-suspended in a small volume of ice-cold Tris buffer, cells were counted and aliquots were frozen at -70°C for later determinations of chlorophyll, metal content and acid-soluble thiol-compounds.

Protein and Chlorophyll determination

Protein was determined by the Biuret method using bovine serum albumin as standard; turbidity was eliminated by centrifugation at 2 000 x *g* for 5 min. Chlorophyll was determined as described by Devars et al. (1998). Heavy metals at the concentrations tested showed no effect on the protein and chlorophyll measurements (Mendoza-Cózatl et al., 2002).

Cadmium, Zinc and Lead determination

Content of heavy metals in acid-digested cells (10^7-10^8 cells) was determined by atomic absorption spectrophotometry in a SpectrAA 640 (Varian Australia Pty Ltd.) spectrophotometer as described by Devars et al. (1998). To determine the amount of Cd^{2+} bound to the external cell surface, half of the harvested cells were washed twice with a chelating buffer containing 50 mM Tris, and 10 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), pH 8.0. The difference in cadmium content between cells washed with and without EGTA was considered as Cd^{2+} bound to the external cell surface. Washing cells with buffer containing 1 mM DTT (DL-dithiotreitol) instead of EGTA yielded similar results.

Bioremoval capacity

To determine the removal capacity of *E. gracilis*, aliquots were withdrawn from the culture medium before addition of the initial cell inoculum and from the cell-free medium after 8 days of culture (*i.e.*, supernatant of the first low-speed centrifugation step during the cell harvesting). Then, the metal content in both samples was determined and the difference was considered as the bioremoval capacity. Negligible

amounts of Cd^{2+}, Zn^{2+} or Pb^{2+} were adsorbed into the glass walls of the culture flasks used in the removal experiments. Since *E. gracilis* lacks a cell wall and negligible amounts of heavy metals are bound to the cell pellicle, bioremoval capacity may also be calculated from the cell growth and metal content in harvested cells. The two above-described approaches yielded similar values.

Content of acid soluble thiol-compounds

Cellular contents of cysteine (Cys), γ-glutamylcysteine (γ-EC), reduced glutathione (GSH) and phytochelatins (PCs) were determined by reverse phase-HPLC coupled to post-column derivation with DTNB (Ellman´s reagent) as described previously (Mendoza-Cózatl and Moreno-Sánchez, 2005). Aliquots of 10^7 cells (in 900 μl) were acidified with 3% perchloric acid (final concentration), mixed vigorously for 30 s and cell debris was removed by centrifugation at 20 000 x *g* for 5 min. All samples were filtered through Millex^{MR}-Millipore filter devices (0.45 μm) prior to analysis by HPLC (50 μl injection loop). Appropriate standards were used to identify the thiol-compounds.

Gel filtration chromatography

A soluble cellular fraction was generated by 3 consecutive freeze-thawing cycles of a suspension of approximately 1 x 10^8 cells in 2 ml of Tris-HCl buffer with 0.2 % Triton X-100, pH 8.0 (saturated with N_2). Cell debris was removed by centrifugation at 20 000 x *g* for 20 min. Soluble fractions from control cells, cells grown in the presence of 50 μM $CdCl_2$, and cells grown with 50 μM $CdCl_2$ plus 200 μM $Pb(NO_3)_2$ were separated in a 1.7 cm x 54 cm Sephacryl S-200 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl pH 8.0 (previously

saturated with N_2) at a flow rate of 0.8 ml/min. Column was calibrated with the standards supplied by Amersham Biosciences: Dextran blue (void volume, 2 000 kDa), Aldolase (158 kDa), Ovoalbumin (43 000), Quimiotripsinogen (25 kDa) and vitamin B_{12} (1.3 kDa). Fractions of 4 ml were collected for determination of metal content and HPLC analysis. Zinc, cadmium and lead were determined by atomic absorption spectrophotometry in samples previously treated with perchloric acid (3 % final concentration) and centrifuged to discard protein. For HPLC analysis, the indicated fractions were pooled and concentrated to a final volume of 1 ml by ultra-filtration through an Amicon filter membrane (Millipore, 30 kDa). The concentrated solution was extensively reduced with 1 mM DTT (final concentration) for 20 min at room temperature, acidified with 3 % perchloric acid, centrifuged and then analyzed by HPLC (200 μl injection loop) for determination of the thiol-compounds as described previously (Mendoza-Cózatl and Moreno-Sánchez, 2005).

Results

Cadmium uptake and removal

E. gracilis growth after 8 days of culture (early stationary growth phase) was significantly diminished by Cd^{2+} concentrations higher than 20 μM (Table 1); at 20 μM or lower Cd^{2+} concentrations no effect on growth was observed. Cell viability was, in all cases, higher than 90% (data not shown). The protein content was not affected by Cd^{2+} concentrations of 5 up to 50 μM (Table 1), whereas a significant increase was found at 100 μM. The Cd^{2+} concentrations used in this work (5-100 μM) did not alter the chlorophyll content. . However, a significant increase in both parameters,

protein and chlorophyll, has been reported at Cd^{2+} concentrations higher that 100 μM (Navarro et al., 1997; Mendoza-Cózatl et al., 2002). Cd^{2+} accumulation was a linear function of the external Cd^{2+} concentration used (Table 1), with no apparent saturation even at 0.5 mM external Cd^{2+}, although cellular density severely decreased (data not shown).

After eight days of culture and at the lower concentrations used (5 and 10 μM $CdCl_2$), *E. gracilis* was able to remove 60% of the Cd^{2+} present in the growth medium, whereas at 20 and 50 μM $CdCl_2$, this value reached 80% (Fig. 1). At this range of concentrations, Cd^{2+} was mainly located inside the cell and not loosely bound to the external surface, since no significant differences were found in the Cd^{2+} content *per* cell by washing them with either 10 mM EGTA (101 $\pm$ 15 nmol $Cd^{2+}/10^7$ cells; mean $\pm$ SD; n = 3; at 50 μM $CdCl_2$) or distilled water (134 $\pm$ 19 nmol $Cd^{2+}/10^7$ cells; n = 3). Washing the cells with 1 mM DTT yielded identical results (not shown). Cd^{2+} removal capacity decreased drastically with Cd^{2+} concentrations higher than 50 μM (Fig. 1). Although the cellular Cd^{2+} accumulation increased concomitantly with the Cd^{2+} concentration in the medium, the cell density was severely affected (Table 1); therefore, the final removal capacity by the overall culture was also affected.

Influence of Zn^{2+} on Cd^{2+} removal

Industrial wastewaters usually contain mixtures of heavy metals (Celebi and Kendir, 2002; Sharaf, 2002). Then, for an organism to be used in bioremediation technology, it is important to determine how a given heavy metal may affect the removal efficiency of a competitor heavy metal. Cd^{2+} and Zn^{2+} are well-known

competitors in several cell processes (Guerinot, 2000; Williams et al., 2000). Thus, to determine the effect of Zn^{2+} on the Cd^{2+} removal capacity of *E. gracilis*, 50 μM $CdCl_2$, was added to the culture medium, as this is the concentration at which *E. gracilis* achieved the optimal Cd^{2+} removal (Fig. 1).

Zn^{2+} at the concentrations tested (5-300 μM) did not affect the cell density or the Cd^{2+} accumulation (Table 2). Protein and chlorophyll content also remained constant (data not shown). Zn^{2+} accumulation was a hyperbolic function of the Zn^{2+} concentration in the culture medium (Table 2); the different patterns found between the Cd^{2+} and Zn^{2+} accumulation behavior (linear and hyperbolic, respectively) suggested that the processes involved in the storage of both heavy metals are different. Zn^{2+} did not affect the Cd^{2+} removal capacity of *E. gracilis*, which remained constant throughout the different Zn^{2+} concentrations assayed (Fig. 2).

On the other hand, Zn^{2+} removal was constant (75 %) up to 50 μM $ZnSO_4$ and decreased significantly at external Zn^{2+} concentrations of 100 and 300 μM (Fig.2). This result was not related to a variation in the cell density, which was not affected by the different Zn^{2+} concentrations (Tale 2). The diminished Zn^{2+} removal capacity was very likely associated with the saturable behavior of the Zn^{2+} accumulation by these cells (Table 2): At $ZnSO_4$ concentrations higher than 100 μM, the amount of metal ion present in the growth media saturated the *E. gracilis* capacity for Zn^{2+} accumulation, which resulted in a decreased capacity in cellular Zn^{2+} removal.

Effect of Pb^{2+} on Cd^{2+} and Zn^{2+} removal

Lead is another heavy metal usually present in industrial wastewaters (Celebi and Kendir, 2002; Sharaf, 2002). In addition, *E. gracilis* tolerates exposure to high concentrations of Pb^{2+} and accumulate part of the metal inside the cell (Navarro et al., 1997; Devars et al., 1998). Therefore, this metal was selected to evaluate the ability of *E. gracilis* to simultaneous remove three different metal ions from the culture medium. In these experiments, the concentrations of Cd^{2+} and Zn^{2+} remained constant in the medium (50 μM each), values at which the removal of both metals was optimal (Figs. 1 and 2).

Remarkably, the two different Pb^{2+} concentrations used did not affect cell growth (Table 3) or protein and chlorophyll content (data not shown). At 100 μM Pb^{2+}, Cd^{2+} accumulation was not affected, whereas at 200 μM Pb^{2+} it significantly decreased (Table 3). In consequence, the ability of *E. gracilis* to remove Cd^{2+} from the culture medium in the presence of 200 μM Pb^{2+} was also affected (Fig. 3). In contrast, Zn^{2+} accumulation and removal were not affected by the presence of Pb^{2+}. These observations suggested that in *E. gracilis* Pb^{2+} and Cd^{2+} might share the same mechanisms for resistance and storage of these heavy metals.

Acid soluble thiols levels under heavy metal exposure

In order to determine whether the glutathione biosynthetic pathway is part of the mechanisms involved in the resistance and storage of Cd^{2+}, Zn^{2+} and Pb^{2+}, the intracellular content of cysteine (Cys), γ–glutamylcysteine (γ-EC), glutathione (GSH) and phytochelatins (PCs) was determined in the presence of the three heavy metals

(Table 4). A typical RP-HPLC profile from a cell extract showing all the thiol-compounds analyzed in this work is shown in Figure 4. Standards were used to identify Cys, γ-EC, GSH, PC_2 and PC_4. The peak corresponding to PC_3 was assigned according to the characteristic chromatographic behavior of this molecule in the usual acetonitrile gradient described in the literature (Rauser et al., 1991). No peaks at 412 nm were observed when the post-column derivation with DTNB was omitted, thus discarding any interference by non-thiol compounds absorbing at this wavelength (Berlich et al., 2002) (data not shown).

Cells grown with 50 μM $ZnSO_4$, without Cd^{2+} or Pb^{2+}, showed similar content of mono-thiols (Cys, γ-EC and GSH) to that previously reported for control cells cultured in the presence of 300 μM $ZnSO_4$ (Table 4; Mendoza-Cózatl et al., 2002), which is the usual concentration present in the Hunter´s medium for *E. gracilis* cultures (Navarro et al., 1997; Devars et al., 1998). With 50 μM Zn^{2+}, the PCs content was below the limit of detection (< 0.1 nmol; Table 4). Addition of 50 μM $CdCl_2$ to the culture medium induced an increase in thiol–compounds: 6-fold for Cys, 27-fold for γ-EC and 10-fold for GSH (Table 4). PCs synthesis was also induced (Table 4), resulting in a thiol-compounds/Cd^{2+} stoichiometry of 3.1; although this is a high ratio, which very likely inactivates Cd^{2+}, 30 % inhibition on cell growth still developed under this condition (Table 2).

Addition of 100 μM Pb^{2+} to the culture medium containing Zn^{2+} and Cd^{2+} brought about a 2-fold increase in the Cys content respect to cells grown in the absence of Pb^{2+} (Table 4), whereas the other thiol-compounds, including the PCs and total thiol content, remained unchanged. Interestingly, the thiol/Cd^{2+} ratio increased

to 4.2 (Tables 3 and 4), but Cd^{2+} accumulation and removal did not vary, suggesting that a fraction of the thiol-compounds was occupied with Pb^{2+}. Moreover, addition of 200 μM Pb^{2+} to the culture medium (+ Cd^{2+} + Zn^{2+}) promoted a significant increase in Cys, GSH and PCs content (Table 4), supporting the idea of an additive effect of high concentrations of Pb^{2+} with Cd^{2+} in activating the GSH and PCs synthesis pathway. In this last condition, the thiol/Cd^{2+} ratio increased up to 7.7, which was apparently in contradiction with the observation that 200 μM Pb^{2+} diminished the ability of *E. gracilis* to accumulate and remove Cd^{2+} from the culture medium (Table 3, Figure 3). Then, this last finding strongly suggested that Pb^{2+} might interfere with the Cd^{2+} storage-inactivation mechanism.

Heavy metal sub-cellular distribution

To further assess the proposed Pb^{2+} competition with Cd^{2+} for the thiol-mediated mechanism of storage-inactivation, the distribution of heavy metals (Zn^{2+}, Cd^{2+} and Pb^{2+}) in the soluble fraction of *E. gracilis* was determined by using gel filtration chromatography (Figure 5, A-C). In control cells (50 μM $ZnSO_4$), Zn^{2+} was mainly located in the high-molecular weight fractions (10-19; > 30 kDa, Fig. 5A). In Cd^{2+} exposed cells, Zn^{2+} was found in both high- and low-molecular weight fractions, but in contrast to control cells, Zn^{2+} was mainly located with the low-molecular weight fractions (25-35; < 30 kDa, Fig. 5B); Zn^{2+} in its free ion form eluted in fraction 37 (not shown). The putative molecules involved in Zn^{2+} chelation were not further characterized in this work, but they are presumably single PCs, which have not been fully polymerized with sulfide.

Cd^{2+} was mainly located in the high-molecular weight fractions in two major peaks (Fig. 5B). When *E. gracilis* was grown in the presence of Zn^{2+}, Cd^{2+} and Pb^{2+} (50 μM, 50 μM and 200 μM, respectively), Zn^{2+} and Cd^{2+} were located in the same two fractions (Fig. 5C) than those from cells grown in the absence of Pb^{2+} (Fig. 5B). Notably, the presence of Pb^{2+} decreased the height of the Cd^{2+} high-molecular weight fractions, in one of which Pb^{2+} was also located (Fig. 5C). When cells were disrupted by sonication, instead of freeze-thawing cycles, the content of Cd^{2+} in the higher-molecular weight fractions (10-19) decreased, whereas that in fractions 25-30, corresponding to lower-molecular weight complexes increased (data not shown). This indicated an unstable, weak binding of Cd^{2+} to the higher-molecular weight fractions.

In an attempt to determine which molecules may be binding simultaneously Cd^{2+} and Pb^{2+}, the fractions containing both heavy metals were analyzed by RP-HPLC. Thus, low-molecular weight thiol-compounds such as Cys, sulfide, GSH, γ-EC and PCs were identified (Fig. 6).

Discussion

Photosynthetic *Euglena gracilis* was able to remove more than 60 % and up to 85 % of the Cd^{2+} added to the culture when grown in the range of 5-50 μM $CdCl_2$ (Fig. 1). This ability did not affect cell viability and, in the case of 50 μM $CdCl_2$, only decreased cell growth by 30 % (Table 1). The IC_{50} values for similar photosynthetic organisms such as *Chlorella vulgaris*, *C. pyrenoidosa*, *Scenedesmus obliquus*, and *S. quadricauda* are in the range of 0.03 to 6 μM $CdCl_2$ (reviewed by Trevors et al., 1996). Thus, these results emphasize the high Cd^{2+} resistance of *E. gracilis*. On the

other hand, in an extensive study made by Matsunaga et al., (1999) where 191 microalgae strains were screened for their potential utilization in bioremediation, it was concluded that only 6 strains of green algae were able to remove 10 % of the cadmium (50 μM) added to the culture medium whereas only 1 (*Chlorella* sp. NKG16014) removed 48 %. These results establish the great potential of successfully applying *E. gracilis* in the bioremediation of heavy metal-polluted water systems in comparison to other photosynthetic microorganisms. Although several groups (Bariaud et al., 1985; Navarro et al., 1997; Devars et al., 1998; Mendoza-Cózatl et al., 2002) have advocated such a proposal, this had not been experimentally demonstrated before.

By considering that the content of protein in the cell is around 50% of the dry weight (Lehninger et al., 2000), it can be estimated that photosynthetic *E. gracilis* is able to accumulate Cd^{2+} up to 0.02 and 0.08 % of its dry weight, at 20 and 50 μM $CdCl_2$, respectively. These values are lower than that reported for Cd^{2+}-hyperaccumulator plants (0.4 %; Ebbs *et al.* 2002) and heterotrophic *E. gracilis* (1.1 %; Avilés et al., 2005), but whether the capacity of *E. gracilis* to remove Cd^{2+} from the culture medium due to the high biomass production is also taken into account, a different picture emerges (Fig. 1; Table 1).

A low concentration factor for a heavy metal exhibited by a given organism may be compensated if the organism generates high biomass. Then, the overall bioremoval capacity of such type of organisms could be similar or higher to that attained by a hyperaccumulator organism with slow growth and poor biomass production. As presented in this work, heavy metal removal is, therefore, the result of both heavy

metal accumulation by cell unit and total biomass in the culture. These two variables, growth and metal uptake/storage, should be considered when a bioremediation strategy is designed and developed. The accumulation factor given by an organism towards a heavy metal is as important as the ability of the same organism to produce high biomass content in the presence of heavy metals.

Wastewater from industries usually contains a mixture of heavy metals (Celebi and Kendir, 2002; Sharaf, 2002). Then it seems relevant to determine how the removal efficiency of an organism towards a given heavy metal may be altered by the presence of other heavy metals. In the case of *E. gracilis*, Zn^{2+} did not affect the Cd^{2+} removal capacity (Fig. 2), and the process involved in accumulation and storage of Zn^{2+} seemed to be independent of the Cd^{2+} accumulation mechanism. This conclusion was supported by (a) the distinct accumulation patterns of Zn^{2+} and Cd^{2+} (saturable and linear, respectively; Tables 1 and 2), (b) the induction of phytochelatins, which were synthesized only with the addition of Cd^{2+} (Table 4) and (c) the different sub-cellular location, as revealed by size exclusion chromatography (Fig. 5).

On the contrary, although *E. gracilis* showed not to be an efficient organism to remove Pb^{2+} from the culture medium, the presence of this metal ion proved to be detrimental for Cd^{2+} removal (Fig. 3). This behavior may be explained in terms of the affinities of thiol-compounds for the three metals, which participate in the Cd^{2+} storage and inactivation cellular mechanism (Clemens, 2001; Mendoza-Cózatl et al., 2005). Pb^{2+} is a weak inducer of PCs (Vatamaniuk et al., 2000); in *E. gracilis* this was evident from the fact that 100 μM Pb^{2+} had no effect on the PCs content (Table

4) and that a higher Pb^{2+} concentration was required to increase the PCs content over that induced by Cd^{2+}.

Phytochelatin induction by Pb^{2+} might be due to an indirect effect of Pb^{2+} on the Cd^{2+}-induced PCs synthesis. For instance, Pb^{2+} and Cd^{2+} have similar dissociation constants for the thiol group, *i.e.*, for GSH, the logarithmic value of the stability constant for Cd^{2+} is 10.5 and for Pb^{2+} is 10.6, whereas that for Zn^{2+} is two orders of magnitude lower at 8.3 (Sillen and Martell, 1971). In consequence, it is conceivable that Pb^{2+} may compete for the already synthesized PCs, thus diminishing their efficiency to bind (and inactivate) Cd^{2+}, whereas Zn^{2+} is unable to compete with Cd^{2+}. In agreement with this proposal, Cd^{2+} and Pb^{2+} in the soluble cellular fraction were located in the same high-molecular weight fractions, in which low-molecular weight thiol-compounds were also present, whereas Zn^{2+} was located in different fractions (Fig. 5). These results do not exclude the possibility that part of the Cd^{2+} and Pb^{2+} content may be also bound to high-molecular weight proteins or that other mechanisms may be acting simultaneously to detoxify the three heavy metals analyzed. However, the results of the present work suggest that thiol-compounds are certainly part of the Cd^{2+} and Pb^{2+} storage-inactivation mechanism in photosynthetic *E. gracilis*. Further work is required to determine and characterize the physicochemical properties of the high-molecular weight complexes containing Pb^{2+} and Cd^{2+} in *E. gracilis*, but data presented in this work support the notion that, as in plants and some yeast such as *Schizosaccharomyces pombe* and *Candida glabrata*, these complexes are essential for an effective Cd^{2+} and Pb^{2+} storage-inactivation process (Speiser et al., 1992; Vande and Ow, 2001).

In Mexico, the permissible concentration of Cd^{2+} in water for agriculture purposes has to be below 3.5 μM (0.4 ppm) (NOM-001-ECOL-1996). Reliable values of heavy metals content in waste water from industries are not often available, but Vázquez-Alarcón et al. (2001) reported Cd^{2+} values of 0.2-1 μM (0.032-0.11 ppm) for water used in food crops irrigation in the Mezquital Valley (Central México); Ni^{2+} and Pb^{2+} were also present in potentially toxic amounts. These observations suggest that the immediate, direct effluent of wastewater from industries to the environment must contain considerable higher amounts of these heavy metals (> 20 μM), which are disposed towards water bodies and soils with the concomitant risk for human health. We have also found high contents of Cd^{2+}, Zn^{2+} and Pb^{2+} in an artificial lake of Mexico City (*Chapultepec* Lake: Cd^{2+}, 5-17 μM; Zn^{2+}, 23-38 μM; Pb^{2+}, 18-49 μM; sampling was made from March 2004 to August 2004) and in a natural lake in the state of Michoacan (*Cuitzeo* Lake: Pb^{2+}, 18-31 µM; Zn^{2+}, 3.6-30 µM; Cd^{2+}, undetectable; sampling was made during July-August, 2004). As photosynthetic *E. gracilis* shows a high capacity for removal of several heavy metals, it is proposed that this microorganism may be á suitable model to apply bioremediation technology on heavy metal polluted water bodies.

It should be emphasized that the efficient and simultaneous removal of three different heavy metals by the protist *Euglena gracilis* was analyzed in a defined culture medium and under axenic conditions. This is still far from the conditions that this and other microorganisms may encounter in a polluted environment such as the effluent of a given industry. In consequence, another issue to resolve for a successful application of microorganisms in bioremediation of heavy metal-contaminated systems is to

determine the capacity of the microorganism to remove heavy metals under more realistic conditions and how external factors such as light/dark cycles, carbon source, pH and temperature variations, essential trace compounds availability (sulfate, vitamin B_{12}) and the presence of other organisms (phytoplankton) may affect this capacity.

Conclusions

The main conclusions of the present work are:

1) *Euglena gracilis* is a suitable organism for simultaneous, relatively fast and efficient Cd^{2+} and Zn^{2+} removal from aqueous media containing concentrations below 100 μM of each heavy metal.
2) In *E. gracilis*, Cd^{2+} and Zn^{2+} do not share the same mechanism of resistance and storage.
3) The presence of Pb^{2+} may be detrimental on the ability of *E. gracilis* to remove Cd^{2+} from an aqueous media. Photosynthetic *E. gracilis* cells are not able to efficiently remove Pb^{2+}.
4) Cd^{2+} and Pb^{2+} may share the thiol-mediated mechanism for resistance and storage in *E. gracilis.*

Acknowledgments

The present work was partially supported by grant No. 43811-Q from CONACyT-México and by **DGAPA-UNAM, PAPIIT-IN215705**.

References

Avilés, C., Loza-Tavera, H., Terry, N., Moreno-Sánchez, R. (2003) Mercury pretreatment selects an enhanced cadmium-accumulating phenotype in *Euglena gracilis*. Arch. Microbiol. 180, 1-10.

Bariaud, A., Bury, M., Mestre, J.C. (1985) Mechanism of Cd^{2+} resistance in *Euglena gracilis*. Physiol. Plant. 63, 382-386.

Berlich, M., Menge, S., Bruns, I., Schimdt, J., Schneider, B., Krauss, G.J. (2002) Coumarins give misleading absorbance with Ellman´s reagent suggestive of thiol conjugates. Analyst 127, 333–336.

Carr, H.P., Cariño, F.A., Yang, M.S., Wong, M.H. (1998) Characterization of the cadmium-binding capacity of *Chlorella vulgaris*. Bull. Environ. Contam. Toxicol. 60, 433-440.

Celebi, S., Kendir, S. (2002) Toxicity assessment of a dye industry treatment sludge. Waste Manag. Res. 20, 541-545.

Clemens, S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486

Coppellotti, O. (1989) Glutathione, cysteine and acid-soluble thiol levels in *Euglena gracilis* cells exposed to copper and cadmium. Comp. Biochem. Physiol. 94C, 35-40.

Devars, S., Hernández, R., Moreno-Sánchez, R. (1998) Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by preexposure to mercury or cadmium. Arch. Environ. Contam. Toxicol. 34, 128-135.

Devars, S., Avilés, C., Cervantes, C., Moreno-Sánchez, R. (2000) Mercury uptake and removal by *Euglena gracilis*. Arch. Microbiol. 174, 175-180.

Dhankher, O.P., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A., Meagher, R.B. (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and γ-glutamylcysteine synthetase expression. Nat. Biotechnol. 20, 1140-1145.

Ebbs, S., Lau, I., Ahner, B., Kochian, L. (2002) Phytochelatin synthesis is not responsible for cadmium tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214, 635-640

Einicker-Lamas, M., Morales, M.M., Miranda, K., Garcia-Abreu, J., Oliveira, A.J.F., Silva, F.L.S., Oliveira, M.M. (2003) P-glycoprotein-like protein contributes to cadmium resistance in *Euglena gracilis*. J. Comp. Physiol. B 173, 559-564.

Guerinot, M.L. (2000) The ZIP family of metal transporters. Biochim. Biophys. Acta 1465, 190-198.

Hamdy, A.A. (2000) Biosorption of heavy metals by marine algae. Curr. Microbiol. 41, 232-238.

Kaplan, D., Heimer, Y.M., Abelovich, A., Goldsbrough, P.B. (1995) Cadmium toxicity and resistance in *Chlorella* sp. Plant Sci. 109, 129-137.

Lehninger A.L., Nelson D.L., Cox M.M. (2000) Principles of biochemistry. 3rd edn., Worth Publishers, Inc. New York 1152 pp. ISBN 1-57259-153-6

Matsunaga, T., Takeyama, H., Nakao, T., Yamazawa, A. (1999) Screening of marine microalgae for bioremediation of cadmium-polluted seawater. J. Biotechnol. 70, 33-38.

Mendoza-Cózatl, D., Devars, S., Loza-Tavera, H., Moreno-Sánchez, R. (2002) Cadmium accumulation in the chloroplast of *Euglena gracilis*. Physiol. Plant. 115, 276-283.

Mendoza-Cózatl, D., Loza-Tavera, H., Hernández-Navarro, A., Moreno-Sánchez, R. (2005) Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, photosynthetic protists and plants. FEMS Microbiol. Rev. *In Press*.

Mendoza-Cózatl, D., Moreno-Sánchez, R. (2005) Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*. Biochim. Biophys. Acta 1706, 88-97.

Navarro, L., Torres-Márquez, M.E., González-Moreno, S., Devars, S., Hernández, R., Moreno-Sánchez, R. (1997) Comparison of physiological changes in *Euglena gracilis* during exposure to heavy metals of heterotrophic and autotrophic cells. Comp. Biochem. Physiol. 116C, 265-272.

Nedelkoska, T.V., Doran, P.M. (2000) Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. Biotechnol. Bioeng. 67, 607-615.

NOM-001-ECOL-1996, Published in *Diario Oficial de la Federación de los Estados Unidos Mexicanos* on January 6, 1997, 31pp.

Rauser, W.E. (1991) Cadmium-binding peptides from plants. Methods Enzymol. 205, 319-333.

Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., Chet, I., Raskin, I. (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13, 468-474.

Sharaf, E.F. (2002) Impact of industrial waste water effluents on mycoflora of the shore sediments of the 3rd oxidation pond, with reference to biosorption of heavy metals. Acta Microbiol. Pol. 51, 293-306.

Sillen, L.M., Martell, A.E. (1971) Stability constants of metal-ion complexes, Spec. Publ. - Chem. Soc. 25, 865 pp.

Speiser, D.M., Abrahamson, S.L., Banuelos, G., Ow, D.W. (1992) *Brassica juncea* produces a phytochelatin-cadmium-sulfide complex. Plant. Physiol. 99, 817-821.

Trevors, J.T., Stratton, G.W., Gadd, G.M. (1996) Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi. Can. J. Microbiol. 32, 447-464.

Tsuji, N., Hirayanagi, N., Okada, M., Miyasaka, H., Hirata, K., Zenk, M.H., Miyamoto, K. (2002) Enhancement of tolerance to heavy metals and oxidative stress in *Dunaliella tertiolecta* by Zn-induced phytochelatin synthesis. Biochem. Biophys. Res. Commun. 293, 653-659.

Vande, W.J.G., Ow, D.W. (2001) Accumulation of metal-binding peptides in fission yeast requires hmt2$^+$. Mol. Microbiol. 42, 29-36.

Vatamaniuk, O.K., Mari, S., Lu, Y.P., Rea, P.A. (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. J. Biol. Chem. 275, 31451-31459.

Vázquez-Alarcón, A., Justin-Cajuste, L., Siebe-Grabach, C., Alcántar-González, G., de la Isla de Bauer, M.L. (2001) Agrociencia 35, 276-274.

Williams, L.E., Pittman, J.K., Hall, J.L. (2000) Emerging mechanisms for heavy metal transport in plants. Biochim. Biophys. Acta 1465,104-126.

Figure Legends

Figure 1. Cd^{2+} removal from the culture medium by *Euglena gracilis*.
Results are mean ± SD of 3-4 independent cell cultures. Significant differences are indicated: a *vs* b ($P<0.01$); b *vs* c ($P<0.005$).

Figure 2. Effect of Zn^{2+} on the Cd^{2+}removal capacity of *E. gracilis*.
$CdCl_2$ was 50 μM whereas Zn^{2+} was varied as indicated. Results are mean ± SD of 3-4 independent cell cultures. Significant differences are indicated: a *vs* b ($P<0.05$); a *vs* c ($P<0.01$); b *vs* c ($P<0.01$).

Figure 3. Effect of Pb^{2+} on Zn^{2+} and Cd^{2+} removal by *E. gracilis*.
$CdCl_2$ and $ZnCl_2$ were 50 μM whereas Pb^{2+} was varied as indicated. Results are mean ± SD of 3 independent cell cultures. Significant differences are indicated: a *vs* b ($P<0.05$); c *vs* d ($P<0.01$).

Figure 4. Representative HPLC profile of thiol-compounds in Cd^{2+}-exposed cells (50 μM).
Thiol-containing compounds were post-column derived with DTNB and detected at 412 nm as described in the Methods section. The retention times for the different compounds were determined with the appropriate standards. Compound marked with an X is unknown but presumably corresponds to PC_5. The inset is an amplification of the peaks corresponding to phytochelatins.

Figure 5. Sub-cellular distribution of accumulated heavy metals. .
The cellular cytosolic fraction was obtained and size exclusion chromatography was performed as described under Material and methods from cells exposed to (A) 50 μM $ZnSO_4$, (B) 50 μM $ZnSO_4$ and 50 μM $CdCl_2$, and (C) 50 μM $ZnSO_4$, 50 μM $CdCl_2$ and 200 μM $Pb(NO_3)_2$. Arrows show the fraction elution for: (1) Dextran blue (void volume, 2 000 kDa), (2) Aldolase (158 kDa), (3) Ovoalbumin (43 kDa), (4)

Quimiotripsinogen (25 kDa) and (5) vitamin B_{12} (1.3 kDa). The chromatograms shown are representative of several ($n > 3$) reproducible experiments.

Figure 6. Thiol-compound composition of the high-molecular weight fractions. Fractions (10-19) from chromatograms similar to that shown in Figure 5C were pooled, concentrated and analyzed by RP-HPLC as described in the Methods section. Compounds marked as X_1 and X_2 were not identified but presumably corresponded to larger chain ($n > 4$) phytochelatins.

Table 1. Effect of Cd^{2+} on cell density, protein, chlorophyll and metal content in photosynthetic *Euglena gracilis*. Cells were harvested, and washed, after 8 days of culture under the indicated conditions. Results are mean ± SD (n) and statistically significant difference ($^{*}P < 0.05$; $^{**}P < 0.01$; Student t-test for non-paired samples), respect to no Cd^{2+} added, is indicated.

	$CdCl_2$ (μM)					
	0	5	10	20	50	100
Cell density (x 10^6 cells/ml)	5.4 ± 0.6 (6)	5 ± 1 (3)	4.6 ± 0.7 (7)	5.3 ± 1 (3)	3.8 ± 0.3* (8)	2.2 ± 0.3** (3)
Protein content (mg protein / 10^7 cells)	6.3 ± 2 (5)	6.5 (2)	6.7 ± 1.2 (4)	7.1 (2)	6.5 ± 1.5 (10)	13.8 ± 5* (4)
Chlorophyll content (μg Chl / 10^7 cells)	55 ± 9 (5)	62 (2)	46 ± 4 (3)	57 (2)	56 ± 5 (7)	66 ± 15 (5)
Cd^{2+} content (nmol / 10^7 cells)	0	6.3 (2)	15 ± 3 (4)	33 ± 5 (3)	102 ± 10 (4)	183 ± 30 (3)

Table 2. Effect of Zn^{2+} on cell density, protein, chlorophyll and metal content in *E. gracilis* after eight days of culture with 50 μM $CdCl_2$. $^{*}P < 0.05$ versus 5 μM Zn^{2+}; $^{**}P < 0.05$ versus 50 μM Zn^{2+}; $^{a}P < 0.01$ versus 5 μM Zn^{2+}; $^{b}P < 0.001$ versus 5 μM Zn^{2+}; $^{c}P < 0.005$ versus 50 μM Zn^{2+}.

	$ZnSO_4$ (μM)				
	5	**20**	**50**	**100**	**300**
Cell density (x 10^6 cells/ml)	3.2 ± 0.3 (3)	2.7 ± 0.3 (3)	3.8 ± 0.7 (3)	3.9 ± 1 (3)	3.2 ± 0.4 (5)
Cd^{2+} content (nmol / 10^7 cels)	125 ± 25 (3)	113 ± 7 (3)	83 ± 6* (3)	90.5 ± 2 (3)	102 ± 10** (4)
Zn^{2+} content (nmol / 10^7 cels)	25 ± 6 (3)	68 ± 15^{a} (3)	130 ± 20^{b} (3)	148 ± 15 (3)	240 ± 30^{c} (5)

Table 3. Effect of lead on cell density, protein, chlorophyll and metal content in *Euglena gracilis* cultured with $CdCl_2$ (50 μM) and $ZnSO_4$ (50 μM). Results are mean ± SD (n). Statistically significant difference ($^*P < 0.01$) respect to cells grown without lead is indicated.

	$Pb(NO_3)_2$ (μM)		
	0	**100**	**200**
Cell density (x 10^6 cells/ml)	3.2 ± 0.4 (4)	2.8 ± 0.2 (4)	3.6 ± 0.4 (4)
Cd^{2+} content (nmol / 10^7 cels)	84 ± 6 (4)	77 ± 17 (4)	54 ± 8* (4)
Zn^{2+} content (nmol / 10^7 cels)	130 ± 20 (4)	144.5 ± 30 (4)	147 ± 21 (3)
Pb^{2+} content (nmol / 10^7 cels)	0	24 ± 7 (4)	31 ± 17 (3)

Table 4 Thiol-containing moleculas in *Euglena gracilis* grown with Cd^{2+}, Zn^{2+} and Pb^{2+}.

Thiol-compounds in cells (10^7 cells) were quantified by RP-HPLC as described under Material and methods. PCs are reported in thiol-content basis as the sum of PC_2, PC_3 and PC_4, identified by stenderds es described by Avilés et al. (2003). Results are mean ± SD of 3 independent cell cultures. ND, not detected. The lower limit of detection was 0.1 nmol. Statistically significant difference ($^{*}P < 0.05$; $^{**}P < 0.01$) respect to cells grown without lead (50 μM $ZnSO_4$ and 50 μM $CdCl_2$) is indicated.

Cells	Cys	γ-EC	GSH	PCs	Total thiols
			nmol / 10^7 cells		
$ZnSO_4$ (50 μM)	10 ± 1.9 (4)	3.5 ± 0.6 (4)	6.9 ± 1.3 (4)	N.D.	21 ± 4 (4)
$ZnSO_4$ (50 μM) + $CdCl_2$ (50 μM)	39 ± 7 (4)	90 ± 10 (4)	61.5 ± 8 (4)	68 ± 11 (4)	258 ± 37 (4)
$ZnSO_4$ (50 μM) + $CdCl_2$ (50 μM) + $Pb(NO_3)_2$ (100μM)	70 ± 18* (3)	112 ± 32 (3)	67 ± 20 (3)	74 ± 22 (3)	323.5 ± 92 (3)
$ZnSO_4$ (50 μM) + $CdCl_2$ (50 μM) + $Pb(NO_3)_2$ (200μM)	58 ± 8* (3)	110 ± 13 (3)	133 ± 15** (3)	118 ± 18* (3)	419 ± 52* (3)

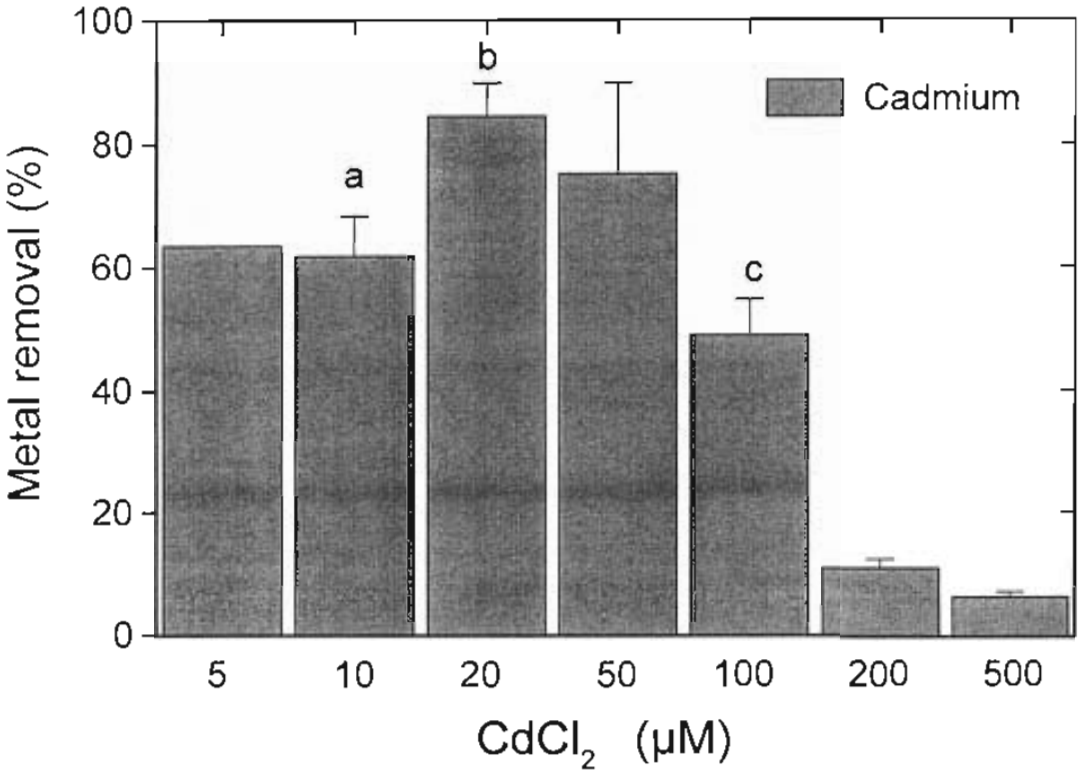

100
80
60
40
20
0
Metal removal (%)
a
b
c
Cadmium
5
10
20
50
100
200
500
CdCl2 (µM)

Figure 1

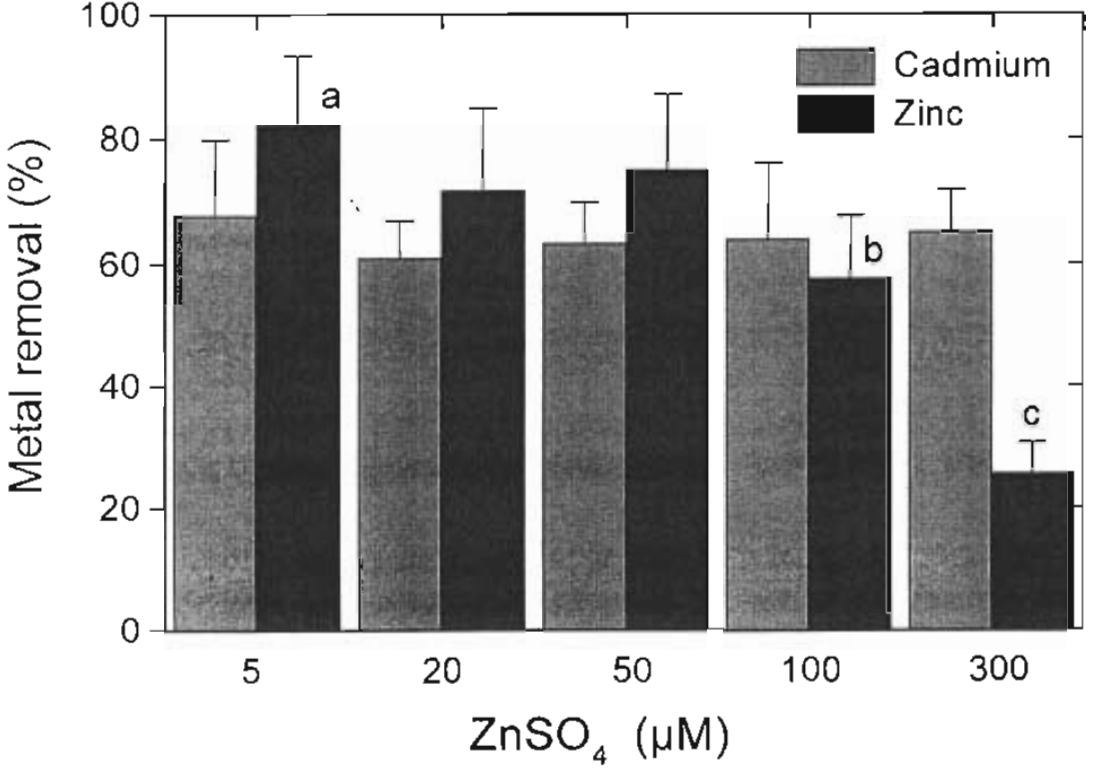

100
80
60
40
20
0
Metal removal (%)
Cadmium
Zinc
a
b
c
5
20
50
100
300
ZnSO4 (µM)

Figure 2

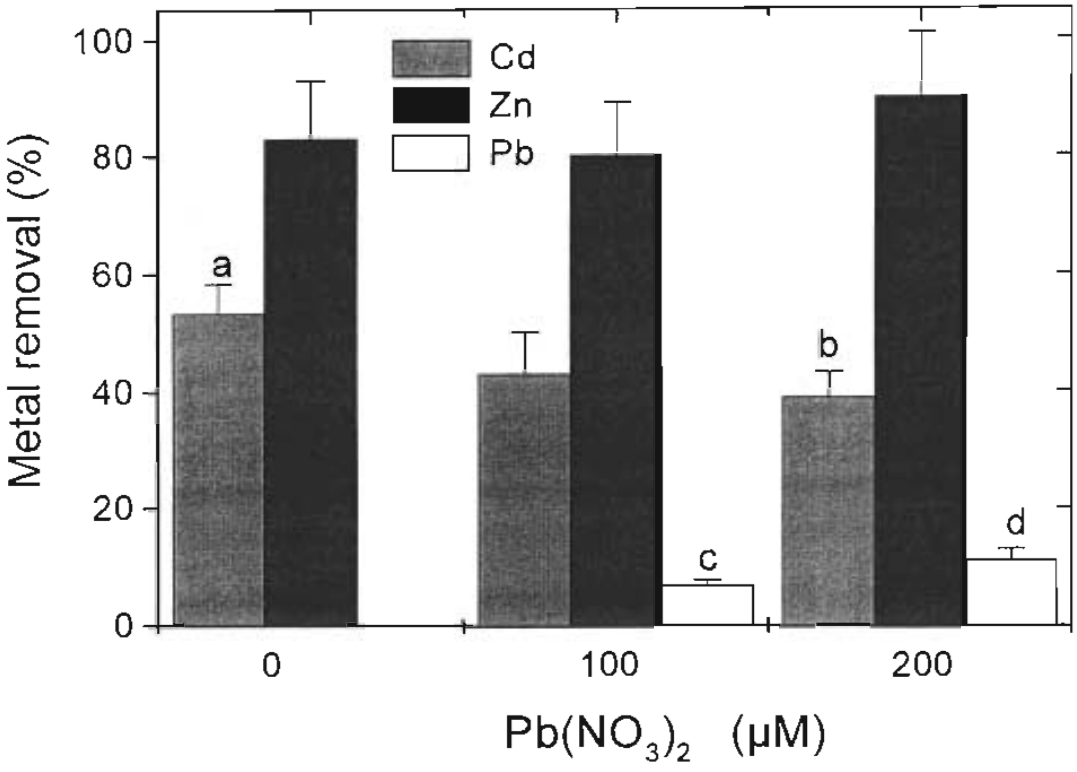


Figure 3

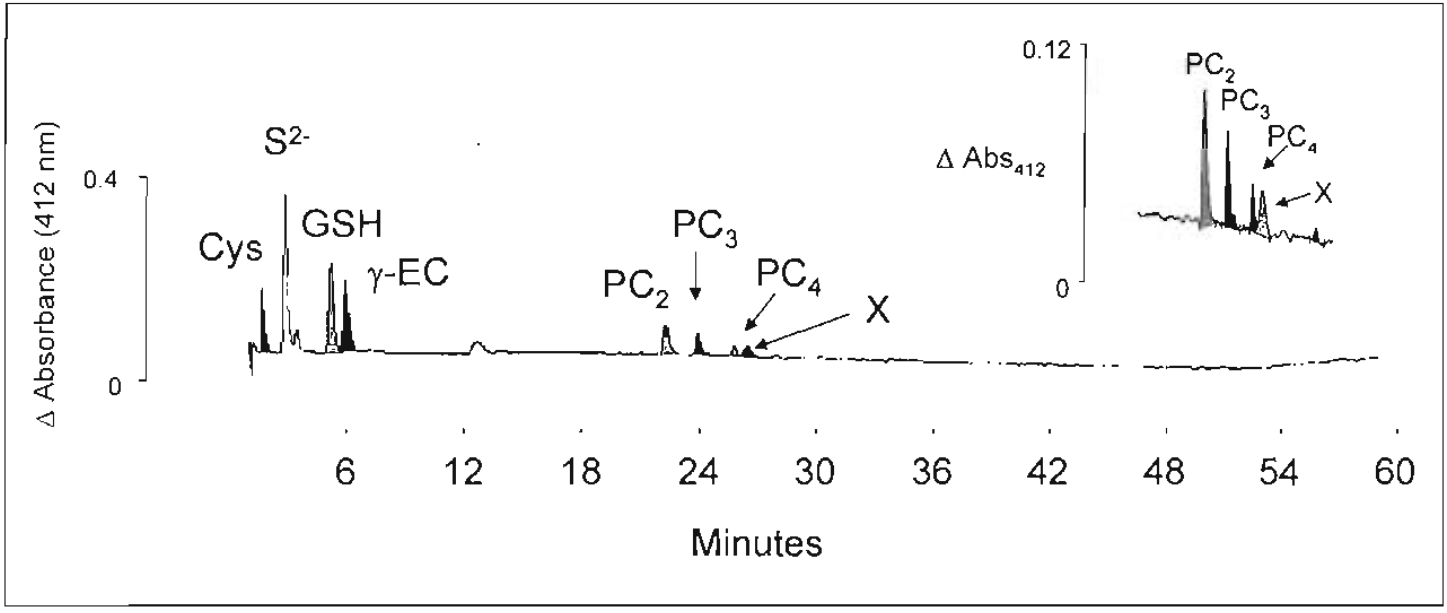

Δ Absorbance (412 nm)
0.4
0
S2-
Cys
GSH
γ-EC
PC2
PC3
PC4
X
6
12
18
24
30
36
42
48
54
60
Minutes
Δ Abs412
0.12
0
PC2
PC3
PC4
X

Figure 4

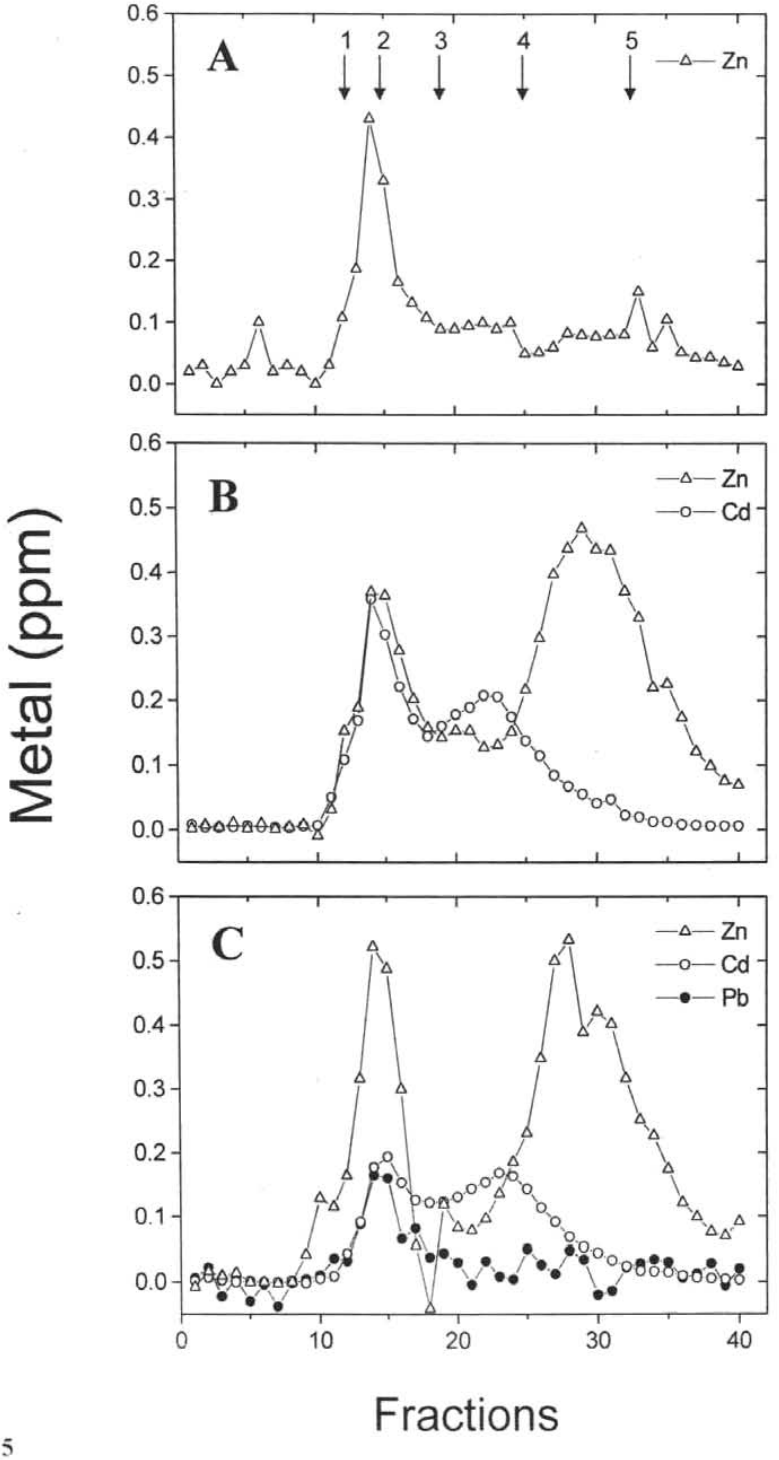

A
B
C
1
2
3
4
5
Zn
Cd
Pb
Metal (ppm)
Fractions
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40

Figure 5

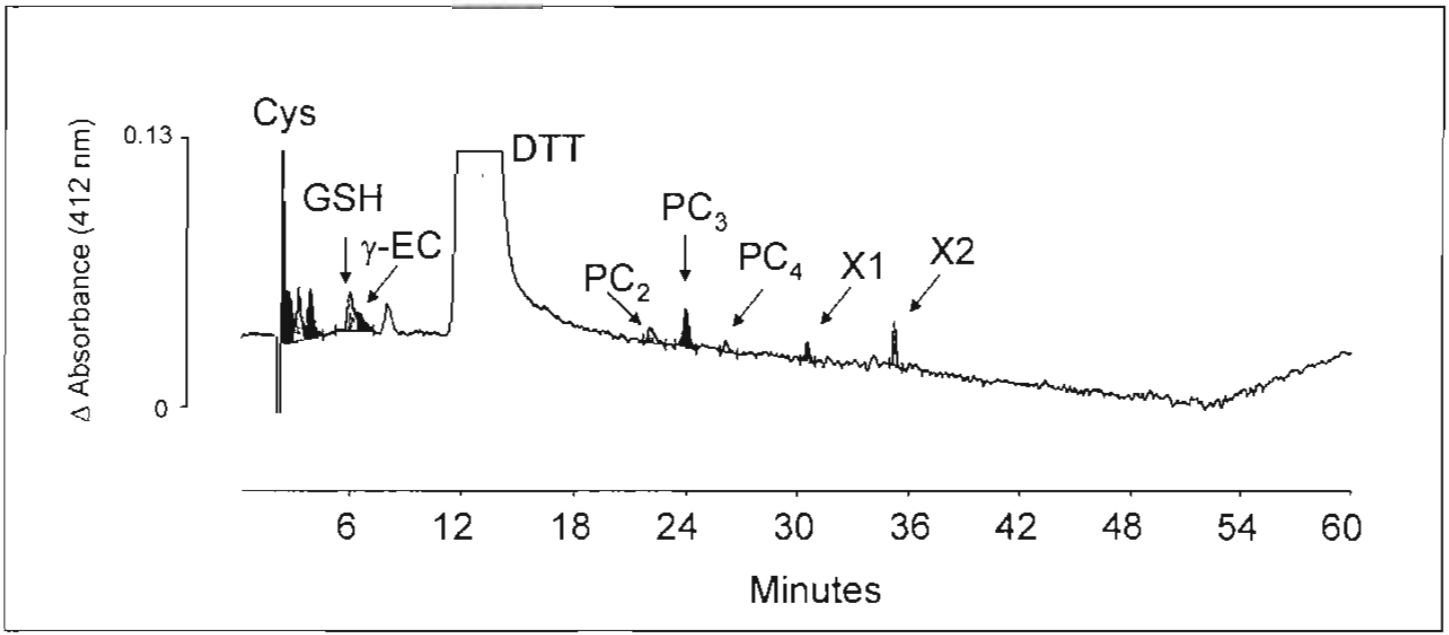

Cys
GSH
γ-EC
DTT
PC2
PC3
PC4
X1
X2
0.13
0
Δ Absorbance (412 nm)
6
12
18
24
30
36
42
48
54
60
Minutes

Figure 6

Capitulo 8. Análisis de control metabólico para la síntesis de glutatión y fitoquelatinas

Los libros de bioquímica y la mayor parte de la literatura enfocada al estudio del metabolismo hacen énfasis en que las vías metabólicas son principalmente controladas por una *etapa limitante*, la cual si es incrementada tendrá como resultado el aumento en el flujo de la vía metabólica en cuestión (Voet y Voet, 1992; Lehninger et al., 2000). Este punto de vista carece de una base sólida y cuantitativa acerca del control que ejerce una enzima sobre el flujo de una vía metabólica determinada.

Durante la década de 1970, dos grupos de investigación europeos desarrollaron una teoría que se conoce actualmente como *teoría de control metabólico* la cual sustituye el término de *etapa limitante* por la determinación cuantitativa del grado de control que una enzima ejerce sobre el flujo de una vía metabólica (Kacser y Burns, 1973; Heinrich y Rapoport, 1974). La formalización de este concepto se denomina *coeficiente de control de flujo*, el cual nos dice qué porcentaje de cambio en el flujo de una vía metabólica se obtendrá al variar infinitesimalmente un porcentaje de la actividad de una enzima. El mismo formalismo se aplica para la concentración de metabolitos. El *coeficiente de control de concentración* nos dice cual será el efecto en la concentración de un metabolito si cambiamos en un porcentaje pequeño la actividad de una enzima dada. Es importante decir que ambos coeficientes están estrechamente relacionados con la cinética enzimática por lo que el diseño experimental para calcular estos coeficientes es posible con los experimentos que comúnmente se realizan en bioquímica.

Si conocemos la estructura de control de una vía, es decir los coeficientes de control y concentración, y los coeficientes de elasticidad, de cada una de las enzimas de una vía metabólica, podemos identificar racionalmente y con bases cuantitativas los sitios

Por otro lado, a pesar de las diferencias intrínsecas en el metabolismo del GSH y FQs entre plantas, levaduras y *Euglena*, el hecho de que la síntesis de GSH en todos los organismos descritos hasta el momento tenga como principal mecanismo de regulación a la retro-inhibición de la primera enzima de la vía (γ-ECS) por GSH (ver capitulo 2), permite extrapolar algunas de las conclusiones del modelo cinético de plantas, a la vía de síntesis de GSH en cualquier otro organismo. Estas conclusiones son:

1.- La síntesis de GSH en condiciones control es mayormente controlada por la demanda (enzimas consumidoras de GSH), mientras que en condiciones de alta demanda (p. ej., exposición a Cd^{2+}), el control de la síntesis de GSH se distribuye entre el bloque productor y el consumidor.

2.- La γ-ECS sólo controla marginalmente el flujo de la síntesis de GSH en condiciones control; la retro-inhibición de la γ-ECS está estrechamente relacionada con la homeostasis del GSH, pero no necesariamente con el control del flujo.

3.- En una vía metabólica se debe hacer una clara distinción entre quien controla el flujo y quien controla la concentración de intermediarios. Dependiendo del estatus celular, el mismo grupo de enzimas puede o no controlar ambas propiedades de la vía.

Capitulo 9. Discusión general

El trabajo realizado en esta tesis demuestra que, respecto al mecanismo de resistencia propuesto: (i) el Cd^{2+} puede entrar al cloroplasto de *Euglena* como ion libre, (ii) la retención del Cd^{2+} dentro del cloroplasto depende de los compuestos tioles y el sulfuro presente en el estroma, (iii) dentro del cloroplasto hay una fitoquelatina sintetasa activa y (iv) el Cd^{2+}, los tioles y el sulfuro forman complejos de alto peso molecular, los cuales inactivan y mantienen fuertemente unido al Cd^{2+}.

En plantas y levaduras esta bien documentado que el Cd^{2+} se acumula principalmente en la vacuola, y puede ser transportado en forma de ion libre usando al intercambiador vacuolar $Ca^{2+}/2H^{+}$ (Salt y Wagner, 1993). *Euglena* carece de una vacuola tipo planta y la mayor parte del Cd^{2+} se acumula en el cloroplasto (Mendoza-Cózatl et al., 2002). La entrada de Cd^{2+} al cloroplasto como ion libre provee una base bioquímica para explicar la acumulación de Cd^{2+} en el cloroplasto, sin excluir la posibilidad de que el Cd^{2+} también pueda ser transportado en forma de complejo GS-Cd ó FQ-Cd (ver capitulo 2).

Se identificaron dos procesos responsables de la entrada de Cd^{2+} al cloroplasto, uno saturable y otro no saturable. Considerando que para otros organismos la concentración estimada de Cd^{2+} libre en el citosol es de 0.005-2 µM (durante la exposición a concentraciones de $CdCl_2$ en el orden de micromolar; Salt y Wagner, 1993) y atendiendo a los parámetros cinéticos de ambos procesos de transporte [*Vm* 11.3 nmol Cd^{2+} min^{-1} (mg proteína)$^{-1}$, *Km* 13 µM para el transportador y 0.13 nmol Cd^{2+} min^{-1} (mg proteína)$^{-1}$ μM^{-1} de la difusión simple], se puede proponer que la entrada de Cd^{2+} al cloroplasto es principalmente mediada por el transportador saturable.

La bioenergética del transporte resultó ser muy diferente a la reportada para plantas y levaduras, en el sentido de no depender de un gradiente electroquímico de H^{+}. Además,

el hecho de que cloroplastos cargados con Cd^{2+} podían rápidamente liberar el metal, involucraba un proceso de difusión facilitada.

Se ha demostrado experimentalmente la difusión facilitada de metales pesados, específicamente Zn^{2+}, en plantas y mamíferos, y los genes responsables del transporte se han agrupado en dos familias de proteínas: CDF (Cation Diffusion Facilitator) y ZIP (Zinc-Iron Proteins). Los proyectos de secuenciación de genomas han permitido identificar genes pertenecientes a estas familias con péptidos de tránsito para organelos, incluido el cloroplasto, pero su caracterización funcional no se ha realizado todavía. Debido a que el transporte de Cd^{2+} al cloroplasto de *Euglena* es mediado por una proteína mediante difusión facilitada, y es inhibido competitivamente por Zn^{2+}, sugerimos que la proteína responsable puede pertenecer a una de estas dos familias de transportadores, CDF ó ZIP. La obtención del gene responsable del transporte será crucial para la asignación final del transportador en una familia determinada.

Una consideración importante sobre el mecanismo de transporte es que la difusión facilitada implica que la dirección en la cual se va a llevar a cabo el transporte depende únicamente de la concentración de Cd^{2+}, en su forma libre, a ambos lados de le membrana del cloroplasto. Este mecanismo no podría explicar la acumulación preferencial del Cd^{2+} dentro del cloroplasto. Sin embargo, si dentro del cloroplasto hay moléculas capaces de secuestrar al Cd^{2+}, la entrada al cloroplasto se vería favorecida, y si además el cloroplasto es capaz de sintetizar *de novo* estas moléculas, se daría una acumulación progresiva del Cd^{2+}. Nosotros demostramos que los tioles orgánicos (Cys, γ-EC, GSH, FQs), junto con el sulfuro inorgánico, son en parte los responsables de secuestrar al Cd^{2+} y por lo tanto favorecer su acumulación dentro del cloroplasto.

Respecto a la síntesis de FQs en *Euglena*, fue muy interesante encontrar síntesis de FQs dentro del cloroplasto. Las repercusiones filogenéticas de este hallazgo resultan ser aún más interesantes. La localización de una secuencia parcial de fitoquelatina sintetasa en una bacteria fotosintética (*Nostoc sp.*) sugiere que el origen de esta enzima pudo ser en el organismo endosimbionte que dio origen al cloroplasto (Tsuji et al., 2004). Esta hipótesis implica que, para el caso de plantas, el gen de la PCS se transfirió del cloroplasto al genóma nuclear y en algún momento de la evolución perdió el péptido de tránsito dando como resultado una enzima citosólica. La peculiar historia evolutiva de *Euglena* (un organismo originalmente no fotosintetico y aparentemente sin el gene de la PCS) y la adquisición del cloroplasto por endosimbiosis secundaria, apoyarían esta hipótesis (Hannaert et al.,2003; Rea et al.,2004).

Las propiedades cinéticas de la PCS de *E. gracilis*, así como la poca eficiencia con la que sintetiza FQs de cadena larga, sugiere que la enzima de *Euglena* se encuentra en un punto intermedio entre el ancestro de la PCS de *Nostoc sp* y la enzima más estudiada de plantas (*A. thaliana*). La obtención de la secuencia de la PCS de *Euglena* será crucial para determinar con certeza que tanto pueden relacionarse estas especulaciones con la historia filogenética de la PCS.

Por otro lado, se ha demostrado que las FQs *per se* no constituyen la totalidad de un mecanismo de resistencia a Cd^{2+}. Las FQs junto con sulfuro y Cd^{2+} deben formar complejos de alto peso molecular para una completa inactivación del Cd^{2+}; la incapacidad de formar estos complejos resulta en una hipersensibilidad al Cd^{2+} respecto a organismos silvestres (Juang et al., 1993; Vande y Ow, 2000). *Euglena gracilis* también forma estos complejos, lo cual sugiere su participación en el mecanismo de resistencia a Cd^{2+} en este organismo.

Si bien los complejos resultaron ser distintos a los reportados en plantas y algunas levaduras respecto a la composición de aminoácidos, péptidos, peso molecular, contenido de monotioles y localización intracelular, la estequiometría $-SH + S^{2-}/ Cd^{2+}$ de 3.4 sugiere fuertemente que estos complejos son los responsables de inactivar al Cd^{2+} dentro de la célula y específicamente, dentro del cloroplasto. Las diferencias entre los complejos de plantas, levaduras y *Euglena* probablemente están relacionadas con las diferentes condiciones en las cuales se forman, vacuola en plantas y levaduras donde el pH puede llegar a 5 y citosol-cloroplasto en *Euglena* donde el pH podría esperarse entre 7 y 8 (Salt y Wagner, 1993; Lehninger et al., 2000). Un pH más alcalino favorecería la unión tiol-Cd, incrementando significativamente la estabilidad de los complejos.

La composición a nivel de péptidos también podría jugar otro papel importante en la estabilidad. Los complejos de plantas y *S. pombe* no contienen monotioles (Cys, γ-EC y GSH) y están formados únicamente por Cd^{2+}, FQs y S^{2-}. Estos complejos son bastante estables una vez aislados, no así los HMWC de *Euglena*, los cuales están constituidos en un 74.8 % por monotioles, y una vez purificados se disocian fácilmente aún en condiciones alcalinas (pH 8). Otro aspecto interesante de los HMWC de *Euglena* fue el alto contenido de aspártico. Se ha sugerido que un análogo del aspartato, sulfinato de cisteína, pudiera estar involucrado en el acarreo de sulfuro hacia los HMWC (Speiser et al., 1992), pero su función no se ha establecido claramente, por lo que todavía no es posible proponer una analogía directa respecto a su función en *Euglena*.

Finalmente, el avance en el conocimiento de los mecanismos de resistencia y acumulación de metales en plantas terrestres para *fitorremediación* es notable; no así el avance en el campo dirigido al tratamiento de aguas que involucra plantas acuáticas y organismos unicelulares como *Euglena gracilis*. Este organismo se ha propuesto como un

candidato para procesos de biorremediación debido a la elevada resistencia y capacidad de acumulación de metales (Devars et al., 1998). Sin embargo, poco se conocía sobre el mecanismo bioquímico de resistencia y acumulación en *Euglena*, así como su verdadera capacidad de remoción de metales de medios acuosos. Esta tesis también abordo este último tema, determinando la capacidad de *Euglena* para remover Cd^{2+} de un medio acuoso bajo diferentes condiciones. Los resultados obtenidos mostraron que *Euglena* es capaz de remover hasta el 80% del Cd^{2+} en condiciones similares a las encontradas en cuerpos de agua contaminados con Cd^{2+} (20-50 µM; Vázquez-Alarcón et al., 2001).

El mecanismo de acumulación de Zn^{2+} pareció no involucrar compuestos tioles y formación de HMWC, aunque tal vez sí comparta con el Cd^{2+} el mecanismo de transporte en el cloroplasto. En cambio, otros metales pesados como el Pb^{2+} sí compartieron el mecanismo de acumulación mediado por tioles, por lo que su presencia en cuerpos de agua podría disminuir la eficiencia de *Euglena* para remover y acumular Cd^{2+}. Sin embargo, los resultados obtenidos aún sostienen que este organismo es un candidato ideal para ser utilizado en procesos de biorremoción de metales de aguas contaminadas, principalmente con Cd^{2+}.

9.1. Conclusiones generales

1.- El Cd^{2+} puede entrar al cloroplasto como ion libre mediante un proceso de difusión facilitada.

2.- El transporte de Cd^{2+} probablemente es mediado por un transportador de Zn^{2+} perteneciente a la familia de transportadores *ZIP* o *CDF*.

3.- La acumulación de Cd^{2+} en el cloroplasto de *Euglena* depende de la cantidad de tioles orgánicos y sulfuro presentes en el estroma.

4.- El 39 % de la fitoquelatina sintetasa se encuentra en el interior del cloroplasto, por lo que las FQs pueden sintetizarse en el cloroplasto de *Euglena gracilis*.

5.- Al igual que plantas y levaduras, la mayor parte del Cd^{2+} y los tioles se encuentran formando complejos de alto peso molecular, los cuales difieren de los de plantas y levaduras en peso molecular composición de péptidos y aminoácidos, pero presentan una similar estequiometría de sulfuro, Cd^{2+} y tioles.

6.- *Euglena gracilis* es capaz de remover hasta el 80 % del Cd^{2+} cuando se cultiva en un medio con 20 ó 50 µM $CdCl_2$.

7.- El zinc en el medio de cultivo no modifica la capacidad de *Euglena* para acumular y remover el Cd^{2+}.

8.- *Euglena* no es un buen modelo para biorremoción de plomo de cuerpos de agua y la presencia del plomo en el medio tiene un efecto negativo en la capacidad de acumulación y remoción de Cd^{2+}.

9.- El Cd^{2+} y el zinc no comparten el mecanismo de resistencia y acumulación mediado por tioles, pero el Cd^{2+} y el plomo probablemente si lo comparten.

Capítulo 10. Perspectivas

Tal vez la pregunta más inmediata que deja abierta esta tesis, y por lo tanto se convierte en una perspectiva, es determinar si las FQs pueden ser transportadas activamente al interior del cloroplasto de *Euglena*. De acuerdo con el esquema de la figura 5.1 (capitulo 5), una posibilidad adicional para explicar la acumulación de FQs en el cloroplasto es que éstas se sinteticen en el citosol y sean internalizadas al cloroplasto mediante un transporte acoplado a ATP. Este mecanismo es independiente del hecho de que las FQs se sinteticen en el interior del cloroplasto y más bien estaría relacionado con una acumulación muy selectiva de estos compuestos dentro de este organelo.

Independientemente de la existencia de este transporte, el mecanismo de internalización es en sí una alternativa para incrementar la capacidad de acumulación de FQs dentro del cloroplasto de *Euglena*. La biología molecular nos ha demostrado que es posible la expresión heteróloga de enzimas, por lo que una hipótesis de trabajo sería que el introducir el gen *hmt1*, responsable del transporte de FQs en *S. pombe*, con un péptido señal dirigido a cloroplasto, se incrementaría la acumulación de FQs en ese organelo.

Una vez mas el análisis de control metabólico (capitulo 8) se hace indispensable para determinar el grado de control de este transportador respecto al flujo hacia FQs y respecto a la concentración de FQs, además de determinar si este transporte no comprometería la concentración de GSH, necesaria para otros procesos celulares (como procesamiento de especies reactivas de oxígeno y detoxificaciòn de xenobióticos). El análisis de control permitirá obtener una visión global de cómo se controla la síntesis de GSH, y en su caso de FQs, en condiciones control y de exposición a Cd^{2+}. Resultados en nuestro grupo de investigación sugieren que el control de la vía en *Euglena* durante la exposición a Cd^{2+} puede ser significativamente diferente a lo esperado para plantas y

levaduras, con una participación muy significativa de la glutatión sintetasa. El análisis de control sentará las bases bioquímicas para entender de manera mecanística esta diferencia.

Adicionalmente, la síntesis de GSH y FQs puede llevarse a cabo tanto en citosol como en cloroplasto (Martínez-Oliver, 2005). El efecto de esta compartamentalización y su relevancia para el control de la vía no se ha evaluado, y será necesaria para el análisis de la vía en el siguiente nivel de organización celular.

Por otro lado, la obtención de la secuencia de la PCS es otra perspectiva importante de este trabajo. Sólo con un análisis filogenético usando las secuencias de la PCS de *Euglena* y de las otras especies reportadas, se podrá tener una idea del origen de esta enzima y de los cambios que ha sufrido durante su historia evolutiva.

Finalmente, es un hecho que se ha avanzado en el conocimiento del mecanismo bioquímico de resistencia a Cd^{2+} en *Euglena gracilis*. Pero el avance en este conocimiento se verá grandemente impulsado cuando las grandes herramientas de la era post-genómica se integren a este estudio: la proteómica, la transcriptómica y la metabolómica. Estas herramientas nos darán un panorama global de como las vías metabólicas en conjunto responden a un estrés como la exposición a metales pesados. Un último reto será además, el encontrar una técnica adecuada para llevar a cabo transformaciones genéticas (introducción y expresión de genes) en protistas fotosintéticos como *Euglena gracilis*, la cual no esta disponible a la fecha.

Referencias

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Plant Physiol 24: 1–15

Albergoni V, Piccinni E, Coppellotti O (1980) Response to hevy metals in organisms-I. Excretion and accumulation of physiological and non physiological metals in *Euglena gracilis*. Comp. Biochem. Physiol. 67C:121-127.

Barbas J, Santhanagopalan V, Blaszczynski M, Ellis WR, Winge DR (1992) Conversion in the peptides coating cadmium:sulfide crystallites in *Candida glabrata*. 48:95-105.

Bariaud A, Bury M, Mestre JC (1985) Mechanism of Cd^{2+} resistance in *Euglena gracilis*. Physiol. Plant. 63:382-386.

Barque JP, Abdelouahab A, Chacun H, Bonaly J (1996) Different heat-shock proteins are constitutively overexpressed in cadmium and pentachlorophenol adapted *Euglena gracilis* cells. Biochem. Biophys. Res. Commun. 223:7-11.

Bonaly J, Bariaud A, Duret S, Mestre JC (1980) Cadmium cytotoxicity and variation in nuclear content of DNA in *Euglena gracilis*. Physiol. Plant. 49:286-290.

Buetow DE (1989) The biology of *Euglena*. Buetow DE (ed.), vol IV Academic press, Inc. 511 pp. ISBN 0-12-139904-4.

Casabó J (1999) Estructura atómica y enlace químico. Editorial Reverté. España 392 pp ISBN 8429171894

Coppellotti O (1989) Glutathione, cysteine and acid-soluble thiol levels in *Euglena gracilis* cells exposed to copper and cadmium. Comp. Biochem. Physiol. 94C:35-40.

Cervantes C, Campos-García J, Devars S, Gutiérrez-Corona F, Loza-Tavera H, Torres-Guzmán JC, Moreno-Sánchez R. (2001) Interactions of chromium with microorganisms and plants. FEMS Microbiol Rev. 25:335-347

Clarkson T (1995) Health effects of metals: a role for evolution. Environ. Health Perspect. 103, 9–12.

Cunningham SD, Ow DW (1996) Promises and prospects of phytoremediation. Plant Physiol. 110:715-719.

Dameron CT, Smith BR, Winge DR (1989) Glutathione-coated cadmium-sulfide crystallites in Candida glabrata. 264:17355-17360.

Dhankher, O.P., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A., Meagher, R.B. (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and γ-glutamylcysteine synthetase expression. Nat. Biotechnol. 20,1140-1145.

Devars S, Hernández R, Moreno-Sánchez R (1998) Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by preexposure to mercury or cadmium. Arch. Environ. Contam. Toxicol. 34:128-135.

Devars S, González-Moreno S, Cañizares-Villanueva O, Sosa-Luna L, Moreno-Sánchez R (1999) Tolerancia a los metales pesados en algas. En: Contaminación ambiental por metales pesados. AGT Editor, México. 157 pp. ISBN 968-463-093-X

De Filippis LF, Hampp R, Ziegler H (1981) The effects of sublethal concentrations of zinc, cadmium and mercury on *Euglena*. Arch. Microbiol. 128:407-411.

Duffus JH (2002) "Heavy Metals"-a meaningless term?. IUPAC technical report. Pure Appl. Chem 74:793-807.

Duret S, Bonaly J, Bariaud A, Vannereau A, Mestre (1986) Cadmium.induced ultrastructural changes in *Euglena* cells. Environ. Res. 39:96-103.

Díaz-Barriga F (1991) Principios de la toxicidad del Cd^{2+}. Ciencia y Desarrollo. 17:61-68.

Díaz-Barriga F, Carrizales L, Calderón J, Batres L, Yañez L, Tabor MUJ, Castelo J. (1995) Measurement of placental levels of arsenic, lead and cadmium as a biomarker of exposure to mixtures. En: Biomonitors and Biomarkers as Indicators of Environmetntal Change: A handbook. Butterworth FM, Corkum LD y Guzmán-Rincón J, eds. Plenum Pub. Co. pp. 139-148.

Ebbs S, Lau I, Ahner B, Kochian L (2002) Phytochelatin synthesis is not responsible for cadmium tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214: 635-640.

Einicker-Lamas M, Morales MM, Miranda K, García-Abreu J, Oliveira AJF, Silva FLS, Oliveira MM (2003) P-glycoprotein-like protein contributes to cadmium resistance in *Euglena gracilis*. J. Comp. Physiol. B 173:559-564.

Falchuk KH, Fawcett DW, Valle BL (1975) Competitive antagonism of cadmium and zinc in the morphology and cell division of *Euglena gracilis*. J. Submicr. Cytol. 7:139-152.

Galvão LAC, Corey G (1987) Cadmio. Serie vigilancia No. 4. Centro Panamericano de Ecología Humana y Saludos, OPS, OMS, Metepec, Estado de México.

Gekeler W, Grill E, Winnacker EL, Zenk MH (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch. Microbiol. 150:197-202.

Gorby MS (1988) Arsenic poisoning [Clinical conference]. West J. Med 149:308-315.

Gray MW (1989) The evolutionary origins of organelles. Trends Genet. 5:294-299

Grill E, Löeffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc. Natl. Acad. Sci. USA 86:6838–6842.

Grill E, Winnacker EL, Zenk MH (1991) Phytochelatins. Methods Enzymol. 205:333-341.

Guerinot ML (2000) The ZIP family of metal transporters. Biochim. Biophys. Acta 1465:190– 198.

Hamdy AA (2000) Biosorption of heavy metals by marine algae. Curr. Microbiol. 41,232-238.

Hannaert V, Bringaud F, Opperdoes FR, Michels PAM (2003) Evolution of energy metabolism and its compartmentation in Kinetoplastida. *Kinetoplastid Biology and Disease* 2:11-41

Hansberg W (2002) Biología de las especies de oxígeno reactivas. En: Mensaje Bioquímico. Cea Bonilla A, del Arenal IP, Riveros Rosas H, Vázquez-Contreras E (eds.). Depto. Bioquímica, Fac. Medicina, UNAM, México. pp. 19-54.

Heiber T, Steinkamp T, Hinnah S, Schwarz, Flügge UI, Weber A, Wagner R (1995) Ion channels in the chloroplast envelope membrane. Biochemistry 34:15906-15917.

Heinrich R, Rapoport TA (1974) A linear steady-state treatment of enzymatic chains. General properties, control and effector strenght. Eur. J. Biochem. 42:89-95

Juang RH, McCue KF, Ow DW (1993) Two purine biosynthetic enzymes required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. Arch. Biochem. Biophys. 304: 392–401.

Kacser H, Burns JA (1973) The control of flux. Symp. Soc. Exp. Biol. 32:65-104.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lagerwerff JV, Specht AW (1970) Contamination of roadside soil and vegetation with cadmium, nickel, lead and zinc. Environ. Sci. Technol. 4:583-586.

Latzko E, Gibbs M (1969) Enzyme activities of the carbon reduction cycle in some photosynthetic organisms. Physiol. Plant 44:295-300.

Lehninger A.L., Nelson D.L., Cox M.M. (2000) Principles of biochemistry. 3rd edn., Worth Publishers, Inc. New York 1152 pp. ISBN 1-57259-153-6

Lozet J, Mathieu C (1991) Dictionary of soil science. 2da ed., A.A. Balkema, Rotterdam. ISBN 9054102012

Marrs KA (1996) The functions and regulation of glutathione-s-transferases in plants. Annu. Rev. Plant. Physiol. Plant. Mol. Biol. 47:127-158.

Martínez-Oliver X (2005) Metabolismo del glutatión en *Euglena gracilis* durante la exposición a Cd^{2+}. Tesis de licenciatura. Facultad de Química. UNAM

Mendoza-Cózatl D, Devars S, Loza-Tavera H, Moreno-Sánchez R (2002) Cadmium accumulation in the chloroplast of *Euglena gracilis*. Physiol. Plant. 115:276-283.

Mendoza-Cózatl D, Moreno-Sánchez R. (2005) Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*. Biochim. Biophys. Acta 1706: 88-97.

Mendoza-Cózatl D, Loza-Tavera H, Hernández-Navarro A, Moreno-Sánchez R (2005) Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, photosynthetic protists and plants. FEMS Microbiol. Rev. *In Press*.

Moreno-Sánchez R, Devars S (1999) Abundancia de los metales pesados en la biosfera. En: Contaminación ambiental por metales pesados. AGT Editor, México. 157 pp. ISBN 968-463-093-X

Moreno-Sánchez R, Díaz-Barriga F, Devars S (1999) Mecanismos de toxicidad y de tolerancia a los metales pesados. En: Contaminación ambiental por metales pesados. AGT Editor, México. 157 pp. ISBN 968-463-093-X

Morris C (1992) Academic press dictionary of science and technology. Academic press, San Diego. 2432 pp. ISBN: 0122004000

Murphy JB, Kies MW (1960) Note on spectrophotometric determination of proteins in dilute solutions. Biochim Biophys Acta 45:382–384.

Nagel, K., Adelmeier, U. and Voigt, J. (1996) Subcellular distribution of cadmium in the unicellular green alga Chamydomonas reinhardtii. J. Plant Physiol. 149: 86–90.

Navarro L, Torres-Márquez ME, González-Moreno S, Devars S, Hernández R, Moreno-Sánchez R (1997) Comparision of physiological changes in *Euglena gracilis* during exposure to heavy metals of heterotrophic and autotrophic cells. 116C:265-272.

Nieboer E, Richardson DHS (1980) The replacement of the nondecsript term "heavy metals" by a biologically and chemically significant classification of metal ions. Environ. Pollut. 1:3-26

Nedelkoska TV, Doran PM (2000) Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. Biotechnol. Bioeng. 67,607-615.

Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333:134-139.

Ohtake H, Cervantes C, Silver S (1987) Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. J. Bacteriol. 169:3853-3856.

Parker SP (1989) McGraw-Hill Dictionary of scientific and technical terms. 4ta ed., McGraw-Hill, New York. 2088 pp. ISBN 0070452709

Pickering IJ, Prince RC, George GN, Rauser WE, Wickramasinghe WA, Watson AA, Dameron CT, Dance IG, Fairlie DP, Salt DE (1999) X-ray absorption spectroscopy of cadmium phytochelatin and model systems. Biochim. Biophys. Acta 1429:351-364

Rea P, Vatamaniuk OK, Rigden DJ (2004) Weeds, worms and more. Papain's long-lost cousin, phytochelatin synthase. Plant Physiol. 136:2463-2474.

Ruotolo R, Peracchi A, Bolchi A, Infusini G, Amoresano A, Ottonello S (2004) Domain organization of phytochelatin synthase. J. Biol. Chem. 279:14686–14693.

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13:468-474.

Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol. 107, 1293–1301.

Salt DE, Wagner GJ (1993) Cadmium transport across tonoplast of vesicles from oat roots. J. Biol. Chem. 268:12297-12302.

Sánchez CL, López MI, Fortoul TL, Saldivar OL, Tovar TA, Antuna BS, Roldán ZA (1994) Inhalación de dos dosis de cloruro de cadmio y modificaiones en la estructura pulmonar. VII Congreso Nacional Estudiantil de Investigación en el Area de la Salud. Gaceta Médica de México.

Satofuka H, Fukui T, Takagi M, Atomi H, Imanaka T (2001) Metal-binding properties of phytochelatin-related peptides. J. Inorg. Biochem. 86:595-602.

Sillen LM, Martell AE (1971) Stability constants of metal-ion complexes. Spec. Publ. - Chem. Soc. 25 , 865 pp.

Speiser DM, Abrahamson SL, Bañuelos G, Ow DW (1992) *Brassica juncea* produces a phytochelatin-cadmium-sulfide complex. Plant Physiol. 99:817-821.

Suzuki, E, Tsuzuki M, Miyachi S (1987) Photosynthetic characteristics of chloroplasts isolated from *Euglena gracilis* Z grown photoautotrophycally. Plant Cell Physiol. 28:1377-1388.

Tsuji N, Nishikori S, Iwabe O, Shiraki K, Miyasaka H, Takagi M, Hirata K, Miyamotoa K (2004) Characterization of phytochelatin synthase-like protein encoded by alr0975 from a prokaryote, Nostoc sp. PCC 7120. Biochem. Biophys. Res. Commun. 315:751-755.

Trevors JT, Stratton GW, Gadd GM (1996) Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi. Can. J. Microbiol. 32, 447-464.

Vande WJG, Ow DW (2001) Accumulation of metal-binding peptides in fission yeast requires $hmt2^{+}$. Mol. Microbiol. 42:29-36.

Vallee BL, Ulmer DD (1972) Biochemical effects of mercury, cadmium, and lead. Annu Rev Biochem 41:91-128

Vázquez-Alarcón A, Justin-Cajuste L, Siebe-Grabach C, Alcántar-González G, de la Isla de Bauer ML (2001) Agrociencia 35:276-274.

Williams LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. Biochim. Biophys. Acta 1465:104–126.

Voet D, Voet JG (1992) Bioquímica. John Wiley & Sons Inc., New York 1315 pp. ISBN 84-282-0906-5

Watanabe M, Henmi K, Ogawa K, Suzuki T (2003) Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic strains of *Euglena gracilis*. Comp. Biochem. Physiol. PartC134:227-234.

Webb M (1972) Binding of cadmium ions by rat liver and kidney. Biochem. Pharmacol. 21:2751-2765.

Weber DN, Shaw FC, Peterin DH (1987) Euglena gracilis Cadmium-binding protein-II contains sulfide ion. J. Biol. Chem. 262:6962-6964.

Williams LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. Biochim. Biophys. Acta 1465:104-126.

Apéndice

ultimate and more stable storage of cadmium. Mutant cells unable to synthesize phytochelatins, transport them or form HMWC are Cd^{2+} hypersensitive in comparison to the wild type organisms (reviewed in Mendoza-Cózatl et al., 2005).

2. Over-expression experiments

The biochemical basis of the Cd^{2+}-inactivation mechanism described above regarding reactions, enzymes and transporters is well known. The cloning of the enzymes involved in the GSH–phytochelatins biosynthetic pathway has prompted several research groups to induce the over-expression of some of these enzymes with the aim of increasing the Cd^{2+}-resistance capacity. Although some of them have had some success, others have not obtained the expected results.

For example, in unstressed conditions (without Cd^{2+} exposure), over-expression of some of the enzymes involved in the sulfate assimilation pathway and Cys synthesis (2–10-fold increase in activity of ATP sulfurylase or O-acetylserine(thiol)lyase in tobacco and serine acetyl transferase in potato chloroplasts) shows little ($\leqslant$2-fold) or no effect on the GSH levels (Hatzfeld et al., 1998; Youssefian et al., 2001; Harms et al., 2002). Likewise, over-expression of the GS by at least 2–3-fold in several organisms does not affect the GSH content (Grant et al., 1997; Zhu et al., 1999b; Creissen et al., 1999; Kim et al., 2003). However, co-expression of γ-ECS (9.1-fold) and GS (18-fold) in *Saccharomyces cerevisiae* induces a 1.8-fold increase in the GSH level, although over-expression of γ-ECS alone also induces similar GSH variation (Grant et al., 1997).

Over-expression of the *Escherichia coli* γ-ECS gene by 50-fold in the cytosol or chloroplast of *Populus tremula* or by 5-fold in the chloroplast of *Brassica juncea* also leads to a significant increase (>4-fold) in the total GSH content (Noctor et al., 1996, 1998). Over-expression of the same gene in tobacco produces a 4-fold elevation in the GSH content and a 40-fold increase in γ-ECS activity in comparison to wild type plants. As in yeast, this indicates that GSH concentration is homeostatically regulated. We shall show that this is mostly due to the feedback inhibition of γ-ECS by GSH, especially under conditions where the demand for GSH controls the flux through GSH. However, in the case of tobacco, the strong over-expression is accompanied by oxidative stress, because apparently the γ-ECS activity exceeds the GS activity, increasing the content of γ-EC and its oxidized form (ESSE) (Creissen et al., 1999). In the same case of tobacco, when γ-ECS and GS are co-expressed, GSH levels increase by 6-fold with respect to non-transformed plants; under these conditions the oxidative damage diminishes and plant growth is partially restored. It is important to note that even after co-expression of γ-ECS and GS, Cys levels are not affected reflecting the great plasticity of sulfate assimilation to an enhanced Cys demand (Noctor et al., 1998; Creissen et al., 1999).

Over-expression of enzymes involved in GSH–phytochelatins synthesis during Cd^{2+} exposure has also been studied. In *S. pombe* the simultaneous over-expression of γ-ECS and GS allows growth in the presence of 1 mM $CdCl_2$ (Kim et al., 2003). Unfortunately, neither phytochelatins concentration or Cd^{2+} accumulation were measured in order to establish whether phytochelatins are responsible for the enhanced Cd^{2+} resistance or whether this is achieved only by the higher GSH levels.

In *B. juncea* over-expression of either GS or γ-ECS during Cd^{2+} exposure increases the Cd^{2+} resistance and accumulation capacity (Zhu et al., 1999a, b). It is again noted that the sulfur assimilation pathway does not limit the increased Cys demand for GSH synthesis. Indeed, enhanced activity of several steps of the sulfate assimilation pathway such as sulfate transporters, ATP sulfurylase, and adenosine 5-phosphosulfate reductase is induced by Cd^{2+} stress (Mendoza-Cózatl et al., 2005). Thus, these results suggest that the best target (or targets) for genetic manipulation to enhance Cd^{2+} resistance and accumulation may be located after Cys synthesis.

Unexpectedly, over-expression of PCS in *A. thaliana* caused hypersensitivity to Cd^{2+}; this phenotype was suppressed when GSH was supplied in the growth medium, suggesting limited intracellular GSH generation (Lee et al., 2003). However, the Cd^{2+} content was not examined. It is possible that added GSH restored growth by reducing the free Cd^{2+} concentration in the culture medium. Natural PCS-defective or phytochelatins vacuole transport-defective strains of *A. thaliana* and *S. pombe* are highly Cd^{2+}-sensitive, which indicates that both proteins are essential for the onset of the cellular mechanisms of heavy metal resistance (reviewed by Mendoza-Cózatl et al., 2005). Indeed, over-expression of heavy metal transporter-1 (HMT1), the protein that catalyses the transport of the complex phytochelatin–Cd into the *S. pombe* vacuole, results in higher Cd^{2+} resistance (Ortiz et al., 1995).

Thus, these observations on enzyme over-expression reveal that the control of the GSH concentration cannot be attributed to only one enzyme, but that it may be distributed among several sites. In consequence, over-expression of only one enzyme of the GSH biosynthetic pathway is not expected to significantly enhance the GSH concentration as so far observed by many researchers. Moreover, the only evaluated parameter has been the GSH concentration, but in no case the flux to GSH has been measured or even considered. This is a common misunderstanding since the *rate-limiting* step concept, often used in the above-mentioned works, does

chemical manipulation, which may enhance the heavy metal removal capacity of organisms.

One of the most studied mechanisms of heavy metal accumulation is the GSH-phytochelatins-mediated Cd^{2+} resistance (Mendoza-Cózatl et al., 2005). Although this mechanism has been described for Cd^{2+}, other heavy metals such as Hg^{2+}, Cu^{2+} and Zn^{2+} may also be inactivated and stored (Devars et al., 1998; Vatamaniuk et al., 2000). Glutathione (GSH) is synthesized by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) (Fig. 1, Scheme 1; Hell and Bergmann, 1988, 1990; Meister, 1995). All γ-ECSs described in bacteria, yeast, plants and animals are physiologically feedback inhibited by GSH with K_i values ranging from 0.1 to 8 mM; for plants such as tobacco and *Arabidopsis thaliana* the K_i values are within a closer range, 0.42 and 0.72 mM, respectively (Hell and Bergmann, 1988; Griffith and Mulcahy, 1999; Jez et al., 2004). This feedback inhibition of γ-ECS by GSH has been considered as the prime regulation mechanism of the pathway (Meister, 1995).

GSH is a ubiquitous molecule with several roles in the cell metabolism such as reactive oxygen species processing, redox state regulation, transport of amino acids, and sulfur storage (Meister, 1995; Noctor and Foyer, 1998). In plants and some yeast such as *Schizosaccharomyces pombe* and *Candida glabrata*, GSH is also used to synthesize phytochelatins (Fig. 1; Scheme 2) (see, for a recent review, Mendoza-Cózatl et al., 2005). Phytochelatins are synthesized by phytochelatin synthase (PCS), which is active when two GSH molecules plus a heavy metal form a thiolate (Cd-GS_2 or Zn-GS_2) and one γ-Glu-Cys moiety is transferred to a GSH free molecule or to a previous synthesized phytochelatin (Vatamaniuk et al., 2000). Once phytochelatins are synthesized, they can be transported into the vacuole and form high molecular weight complexes (HMWC) around a CdS crystallite core. These HMWC are the

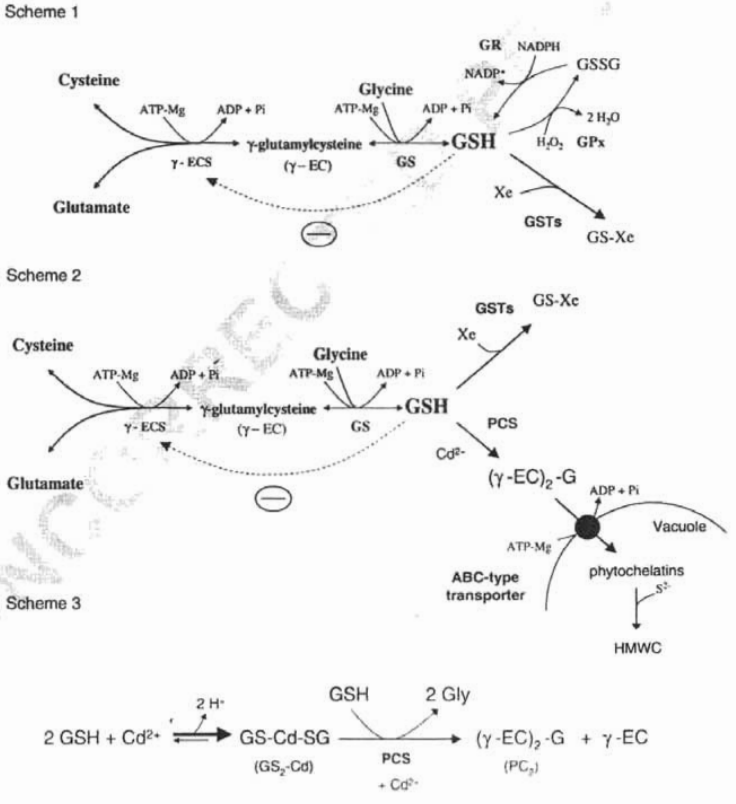


Fig. 1. GSH metabolism under control (Scheme 1) and cadmium exposure (Schemes 2 and 3). Scheme 3 shows the explicit reaction of phytochelatin synthase. HMWC, high molecular weight complexes.

3B2v8.06a/w (Dec 5 2003).51c XML:ver:5.0.1 YJTBI : 4061 Prod.Type:FTP pp.1–18(col.fig.:NIL) ED:Jyoti PAGN:sree SCAN:Roopa

ARTICLE IN PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Journal of Theoretical Biology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Journal of Theoretical Biology

www.elsevier.com/locate/yjtbi

Control of glutathione and phytochelatin synthesis under cadmium stress. Pathway modeling for plants

David G. Mendoza-Cózatl, Rafael Moreno-Sánchez*

Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano 1, Sección XVI Tlalpan, 14080 México D.F., México

Received 31 August 2004; received in revised form 30 June 2005; accepted 7 July 2005

Abstract

Glutathione (GSH) plays several roles in cell metabolism such as redox state regulation, oxidative stress control, and protection against xenobiotics and heavy metals. GSH is synthesized in two steps catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase. γ-ECS is feedback inhibited by GSH, which has lead to the proposal that this enzyme acts as the rate-limiting step in the pathway.

Thus far, the study of GSH metabolism has been confined to GSH synthesis (GSH supply), without considering the GSH-consuming enzymes (GSH demand). Several works have shown that the demand block of enzymes may have a significant control on a pathway; therefore, we hypothesize that GSH-consuming enzymes may exert some control on GSH synthesis.

A kinetic model of GSH and phytochelatin synthesis in plants was constructed using the software *GEPASI* and the kinetic data available in the literature. The main conclusions drawn by the model concerning metabolic control analysis are (1) γ-ECS is indeed a rate-limiting step in GSH synthesis, but only if GSH-consuming enzymes are not taken into account. (2) At low demand, GSH-consuming enzymes exert significant flux-control on GSH synthesis whereas at high demand, supply and demand blocks share the control of flux. (3) In unstressed conditions, flux to GSH is controlled mainly by demand, so that γ-ECS determines the degree of homeostasis of the GSH concentration. Under cadmium exposure, the GSH demand increases and flux-control is re-distributed almost equally between the supply and demand blocks. (4) To enhance phytochelatins synthesis without depleting the GSH pool, at least two enzymes (γ-ECS and PCS) should be increased and/or, alternatively, a branching flux (GSH-S-transferases) could be partially diminished.

Keywords: Metabolic control analysis; γ-glutamylcysteine synthetase; Glutathione synthetase; Phytochelatin synthase; Glutathione S-transferases; Supply–demand analysis; Universal method

1. Introduction

One potential strategy to efficiently remove heavy metals from contaminated sites is the use of organisms with high heavy metal resistance and accumulation capacity (Salt et al., 1995; Dhankher et al., 2002). This strategy, called "bioremediation", has several advantages over physical remediation methods in the sense of costs, practice and the scale at which the processes operate (Salt et al., 1995; Dhankher et al., 2002). Thus, the understanding of the biochemical mechanisms related to heavy metal resistance and accumulation may lead to the identification of targets for genetic and

Abbreviations: C_E^M, concentration control coefficient of a given enzyme (E) on the metabolite M; C_E^J, flux control coefficient of a given enzyme (E) on flux (J); ε_S^E, elasticity coefficient of a given enzyme (E) towards a metabolite (S); γ-EC, γ-glutamylcysteine; ESSE, γ-glutamylcysteine oxidized; γ-ECS, γ-glutamylcysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GS, glutathione synthetase; GSSG, oxidized glutathione; GSTs, glutathione S-transferases; HMWC, high molecular weight complexes; MRP, multi-drug resistance protein; PCS, phytochelatin synthase; pcs, phytochelatins; Xe, Xenobiotic

*Corresponding author. Tel.: +5255 5573 2911; fax: +5255 5573 0926.

E-mail addresses: dgmcozatl@yahoo.com.mx (D.G. Mendoza-Cózatl), rafael.moreno@cardiologia.org.mx, morenosanchez@hotmail.com (R. Moreno-Sánchez).

0022-5193/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jtbi.2005.07.003

not distinguish between metabolite concentration control and flux control through the pathway.

2.1. In silico experimentation

The over-expression results, together with the feedback inhibition of γ-ECS by GSH have led to consider this enzyme as the *rate-limiting* step of the pathway. One of our goals in the present work is to replace this misleading concept by the appropriate framework of metabolic control analysis (MCA; see below). In addition, it has been experimentally demonstrated in other systems that significant flux control may reside outside a synthetic block of enzymes, i.e. in the consuming block of enzymes (Rodríguez-Enríquez et al., 2000; Koebmann et al., 2002). The demand block of enzymes has not yet been considered when GSH synthesis has been studied.

Several metabolic pathways of potential biotechnological and clinical relevance such as glycolysis in erythrocytes (Heinrich et al., 1977), *Trypanosoma brucei* (Bakker et al., 1997, 1999), and yeast (Teusink et al., 2000; Pritchard and Kell, 2002); oxidative phosphorylation in skeletal muscle (Korzeniewski, 1998); threonine synthesis in *E. coli* (Chassagnole et al., 2001); and Calvin cycle (Poolman et al., 2000) have been modeled in the last few years. In some of these efforts, pathway simulation has led to further experimentation to assess its flux control predictions.

In the present work, we have constructed a kinetic model of the GSH and phytochelatins synthesis in an attempt to understand the control structure of the pathway applying the concepts of MCA (for a detailed review, see Fell, 1997). This analysis establishes a theoretical framework that may help to explain the results obtained with the enzyme over-expression experiments. It may also help to identify and design strategies to improve the Cd^{2+}-accumulating ability of resistant organisms. MCA shows that it is feasible to quantitatively determine the degree of control exerted by each site of a given pathway. In addition, it provides the different experimental designs to achieve such a goal, and directs to what has to be measured to establish why an enzyme exerts significant or negligible control. This approach has shown that, in most cases, the flux in a metabolic pathway is not necessarily controlled by only one enzyme, the putative *rate-limiting* step, but the control can be distributed among several enzymes of the pathway. It has also been observed that the distribution of flux control may change depending on the external conditions, and that the main controlling steps may change between organisms (Fell, 1997; Moreno-Sánchez et al., 1999; Rossignol et al., 2000).

MCA also establishes that, instead of embarking on a "trial and error" sequence of experiments for identifying the ill-conceptualized "rate-limiting step", the control profile of a pathway should be evaluated systematically. Control is quantified in terms of flux-control (C_E^J) and concentration-control (C_E^M) coefficients (the degree of control exerted by a given enzyme E on flux J and metabolite M concentration, respectively) (Fell, 1997). The control coefficients are system properties that are in turn determined by the elasticity coefficients (the sensitivity of a given enzyme rate to variations in one of its ligands under particular steady-state conditions). Some enzymes have elasticities to factors other than their own substrate, product or enzyme concentration such as allosteric modulators.

3. Description of the model

The kinetic model is based on data obtained from GSH synthesis in tobacco cell suspension cultures during illumination and was simulated using the computer program *GEPASI* (available at http://www.gepasi.org; Mendes, 1993). Identical results were obtained by using the software *PySCeS* (http://pysces.sourceforge.net; Olivier et al., 2005). GSH-synthesizing enzymes (γ-ECS and GS) were from the same source (*Nicotiana tabacum*) and characterized by the same group (Hell and Bergmann, 1988, 1990). The reaction stoichiometry, the kinetic constants and the fixed metabolites are shown in Tables 1–3, with the maximal rate values (V) calculated from the activities in the crude cell extracts instead of the activities from the purified enzymes. For a detailed explanation of the stoichiometry reactions and the kinetic equations used see Appendix A. Two models were built: (1) in control, unstressed conditions (Fig. 1, Scheme 1) and (2) under cadmium exposure (Fig. 1, Scheme 2 and 3).

Glutathione S-transferases (GSTs) were chosen as the GSH-consuming block because they are the only enzymes that effectively consume GSH by forming conjugates with electrophilic compounds (Fig. 1, Scheme 1). Kinetic constants of GSTs were also taken from tobacco (Droog et al., 1995). Glutathione peroxidase (GPx) is another enzyme that uses GSH to reduce organic peroxides forming GSSG (Fig. 1, Scheme 1). Under unstressed conditions glutathione reductase (GR) is able to maintain a high GSH/GSSG ratio at expenses of NADPH (up to 100/1; Meister, 1995). Therefore, this branch involving a moiety-conserved cycle may have no relevant control on GSH synthesis (see Hofmeyr et al., 1986 for control analysis of moiety-conserved cycles); for this reason, this branch was not included in the model. However, under conditions in which the redox state of the cell is compromised, this set of reactions may be relevant for the control of GSH synthesis.

In the cadmium exposure model, the GSH-consuming block of enzymes involves the GSTs and PCS activities. PCS has been found in tobacco (Nakazawa et al., 2002).

but a detailed description of kinetics has been made for the *A. thaliana* enzyme (Vatamaniuk et al., 2000, 2004). Kinetic parameters for phytochelatins synthesis were then taken from the *Arabidopsis* studies. Although compartmentation of phytochelatins into the vacuole is well described for higher plants, phytochelatins transport has only been determined in tonoplasts isolated from oat roots (Salt and Rauser, 1995). Therefore, kinetics for phytochelatins transport was taken from this last organism. A more accurate estimation of the control distribution of the phytochelatins synthesis pathway will be possible when the kinetic parameters of the enzymes and transporters from the same organism become available. Nonetheless, we expect that the conclusions reached with the cadmium exposure model are relatively insensitive to the inclusion of kinetic parameters from a different biological source.

The volume (ml) for compartments was 1 for cytosol and 0.8 for vacuole. These values were chosen to maintain the volume proportionality found in most plant cells (Lehninger et al., 2000). ATP was assumed to be present at saturating concentrations and, therefore, was not included in the model (see Appendix A).

3.1. Kinetic equations

Kinetic equations were taken from the kinetic characterization reported for each enzyme. For γ-ECS

Table 1
Reactions of glutathione and phytochelatin synthesis

Enzyme	Reaction represented in *GEPASI*
(1) *Unstressed conditions*	
γ-ECS[a]	Glu + Cys = γ-EC
GS[a]	γ-EC + Gly = GSH
GSTs	GSH + Xe → GS-Xe
(2) *Cadmium exposure*[b]	
PCS[a]	GSH + GS_2-Cd → PC_2 + γ-EC + Cd^{2+} + 2Gly
HMT1	PC_2 → Pcin
Non-enzymatic formation of GS_2-Cd	2GSH + Cd^{2+} = GS_2-Cd

[a]See Appendix for justification on the stoichiometry of the reactions.
[b]Including the reactions showed for unstressed conditions.

Table 3
Fixed metabolites of the model

Metabolite (mM)	Unstressed conditions	Cadmium exposure
Glu	5.25	5.25
Cys	0.3	1
Gly	1	1
Xe	0.0003	0.0003
GS-Xe	1	1
Cd^{2+}	N.A.	0.01[a]
PCin	N.A.	1

N.A., non-applicable.
[a]To maintain a constant phytochelatin synthesis, cadmium was initialized at 0.01 mM but not fixed, because due to its high affinity for GSH it would deplete the GSH pool. Almost all cadmium (>99%) remained in the form of GS_2-Cd, which is the true substrate of PCS (Vatamaniuk et al., 2000). In the following reaction with PCS, Cd^{2+} is recycled (see Table 1).

Table 2
Kinetic parameters of the model

Enzyme or reaction	$V^{a,b}$	K_a (mM)	K_b (mM)	K_p (mM)	K_i (mM)	K_{eq}
Unstressed conditions						
γ-ECS	1.73	10.4	0.19	0.3[c]	0.42	5597[d]
GS	1.14	0.022	0.3	0.03[c]	N.A.	5597[d]
GSTs	103[e]	0.4	0.5	N.A	N.A.	N.A.
Cadmium exposure						
γ-ECS	4.32[f]	10.4	0.19	0.3	0.42	5597
GS	2.85	0.022	0.3	0.03	N.A.	5597
PCS	3.25	13.6	0.0092	N.A.	N.A.	N.A.
Phytochelatins transporter	1	0.033	N.A.	N.A.	N.A.	N.A.
2GSH + Cd^{2+} = GS_2-Cd	N.A.	N.A.	N.A.	N.A.	N.A.	2.23×10^{15} M^{-2}

[a]V units are: nmol min^{-1} (mg protein)$^{-1}$. N.A. Non-applicable.
[b]Velocities were calculated from the rates found in extracts at non-saturating substrate concentrations and by using the kinetic mechanism described for each enzyme (see *Description of the model* section). Data were taken from Hell and Bergmann (1988) (γ-ECS); Hell and Bergmann (1990) (GS); Droog et al. (1995) (GSTs); Löeffler et al. (1989) and Vatamaniuk et al. (2000) (PCS); and Salt and Rauser (1995) (pcs transport); data for GS_2-Cd stoichiometry was compiled by Vatamaniuk et al. (2000).
[c]There are no experimental data for K_p values of γ-ECS and GS; see Appendix for K_p estimation.
[d]K_{eq} was calculated using the $\Delta G'^{o}$ for a peptide bond formation at expenses of ATP hydrolysis (see also Appendix).
[e]This value represents an average of activities under unstressed conditions described for several plant species (Irzyk and Fuerst, 1993; Droog et al., 1995; Riechers et al., 1997; DeRidder et al., 2002).
[f]In the cadmium exposure model, γ-ECS and GS rate activities were increased by a factor of 2.5, which is an increase average found for both enzymes after cadmium exposure.

the rate equation used was (1), a reversible Michaelis–Menten random bi-reactant mechanism with competitive inhibition by GSH against glutamate (Hell and Bergmann, 1990; Griffith and Mulcahy, 1999),

$$v = \frac{V/K_aK_b(AB - P/K_{eq})}{1 + A/K_a + B/K_b(1 + I/K_i + A/K_a) + P/K_p + I/K_i} \quad (1)$$

in which V is the maximal rate of the forward reaction; A = Glu, B = Cys, P = γ-EC, I = GSH; K_a, K_b, K_p, and K_i are the respective dissociation constants for each ligand, and K_{eq} the equilibrium constant of the overall reaction.

For GS the rate equation used was, a reversible Michaelis–Menten random bi-reactant mechanism (there is not yet a kinetic mechanism described for GS; kinetic data are from Hell and Bergmann, 1988):

$$v = \frac{V/K_aK_b(AB - P/K_{eq})}{1 + A/K_a + B/K_b(1 + A/K_a) + P/K_p} \quad (2)$$

with A = γ-EC, B = Gly, and P = GSH.

For GSTs the rate equation used was, an irreversible Michaelis–Menten random bi-reactant mechanism (Droog et al., 1995), in which the binding of one substrate does not affect the affinity of the other substrate (Segel, 1975):

$$v = \frac{V(AB/K_aK_b)}{1 + A/K_a + B/K_b + AB/K_aK_b} \quad (3)$$

in which A = GSH and B = Xenobiotic.

For PCS the rate equation used was, an irreversible ping-pong mechanism (Segel, 1975; Vatamaniuk et al., 2000):

$$v = \frac{VAB}{K_bA + K_aB + AB}, \quad (4)$$

where A = GSH and B = GS_2–Cd.

For phytochelatins transport into vacuoles the rate equation used was, irreversible Michaelis–Menten (Salt and Rauser, 1995):

$$v = \frac{V(A/K_a)}{1 + (A/K_a)} \quad (5)$$

in which V is the maximal rate of the forward reaction, A = phytochelatin-2 and K_a the dissociation constant for phytochelatins. Although this transport is ATP dependent, the reaction was considered uni-reactant because the K_{miATP} is very low (0.2 mM; Salt and Rauser, 1995) and ATP values in the plant cell are usually above 0.7 mM (Geingenberger et al., 2001; van Dongen et al., 2003). Then, under physiological conditions ATP would not apparently be a limiting factor for phytochelatins transport.

It is important to recall that the C_E^J is a quantitative measurement of the flux sensitivity to the concentration (or, more strictly, to the activity) of one particular enzyme. C_E^J directly reflects the impact on flux by changing an enzyme activity caused by biochemical factors (biosynthesis, degradation, external activation or external inhibition). Thus, a C_E^J of 1 implies total control of that enzyme over the flux, as expected for a rate-limiting step; on the other hand, a C_E^J of zero means that this enzyme exerts no control over the pathway flux. Similarly, the C_E^M is a quantitative measurement of the dependence of a metabolite concentration on the activity of one particular enzyme; metabolite and enzyme may or may not have to establish an obligatory interaction to generate a C_E^M, but they have to be interconnected by a pathway. An absolute value of C_E^M of 1 or higher means that that enzyme exerts significant control on the concentration of a given metabolite and a C_E^M of zero means that the activity of that enzyme exerts no control on the concentration of such metabolite. A large C_E^M value reflects the high sensitivity of metabolite concentration to enzyme activity.

4. Results

4.1. Steady-state properties of the GSH synthesis model

GSH synthesis was successfully modeled with the kinetic data available in the literature and using the software *GEPASI*. We initially modeled GSH synthesis using fixed concentrations of substrates with values similar to those reported for plants (Table 3), leaving the two crucial intermediaries, γ-EC and GSH, as variables of the system. Remarkably, the steady-state concentrations of these intermediaries predicted by the modeling were in good agreement with those reported in the literature (Table 4).

The flexibility of the model was assessed to establish the range of stable responses to a given stimulus. Thus, we initially analysed the effect of small, physiological variations in the concentration of several essential metabolites (Glu, Cys, Gly and Xe; Noctor et al., 1997, 1998) on the flux to GSH (J_{GSH}) and the GSH concentration (Fig. 2). The arrows shown in Fig. 2 represent the physiological concentration of each substrate reported in plants (poplar and tobacco) (Hell and Bergmann, 1990; Noctor et al., 1997, 1998).

Variation in Glu concentration by a factor of 2 at both sides of the scale (3–12 mM) has no effect on J_{GSH}; only at Glu values below 1 mM, J_{GSH} is affected. As Glu is a substrate of the first enzyme (γ-ECS), then the decrease in J_{GSH} is related to the decrease in the γ-ECS rate due to substrate limitation. However, this effect has a poor physiological relevance since Glu concentrations are usually higher than 1 mM in plants (Hell and Bergmann, 1990; Noctor et al., 1998). On the contrary, small changes in Glu concentration have a strong effect on GSH concentration (Fig. 2A). Glu is not usually

Table 4
Steady-state concentrations of intermediaries of GSH and phytochelatin synthesis obtained by modeling and compared with those reported in the literature

Metabolite	Unstressed conditions	Cadmium exposure				Literature
		No changes[a]	γ-ECS (5 ×) (mM)	PCS (5 ×) (mM)	γ-ECS + PCS (2.5 ×) (mM)	
γ-EC	0.04	0.085	1.13	0.087	0.54	0.01–0.5[b]
GSH	3.4	1	2.85	0.33	1	1–10[c]
Phytochelatins (cytosol)	N.A.	0.008	0.03	0.02	0.036	0.03–8[d]

Variable metabolites were initialized at 0.001 mM.N.A., non-applicable.
[a]In the cadmium exposure model, the maximal rate of γ-ECS and GS was increased 5-fold respect to unstressed conditions.
[b]Determined in plants in unstressed conditions (Noctor et al., 1997, 1998); for metabolite content, a volume of 210 μl/g fresh weight was used (Noctor et al., 1997).
[c]Determined in unstressed conditions for different animal cells and tissues (Meister, 1995), plants (Noctor and Foyer, 1998) and *Euglena gracilis Z* (Mendoza-Cózatl et al., 2002).
[d]The lowest value represents the concentration of the smallest phytochelatin ($n = 2$), and the highest the sum of all phytochelatins, both in the whole cell; *Euglena gracilis Z* grown for 10 days with 30 μM $CdCl_2$ (Gekeler et al., 1988).

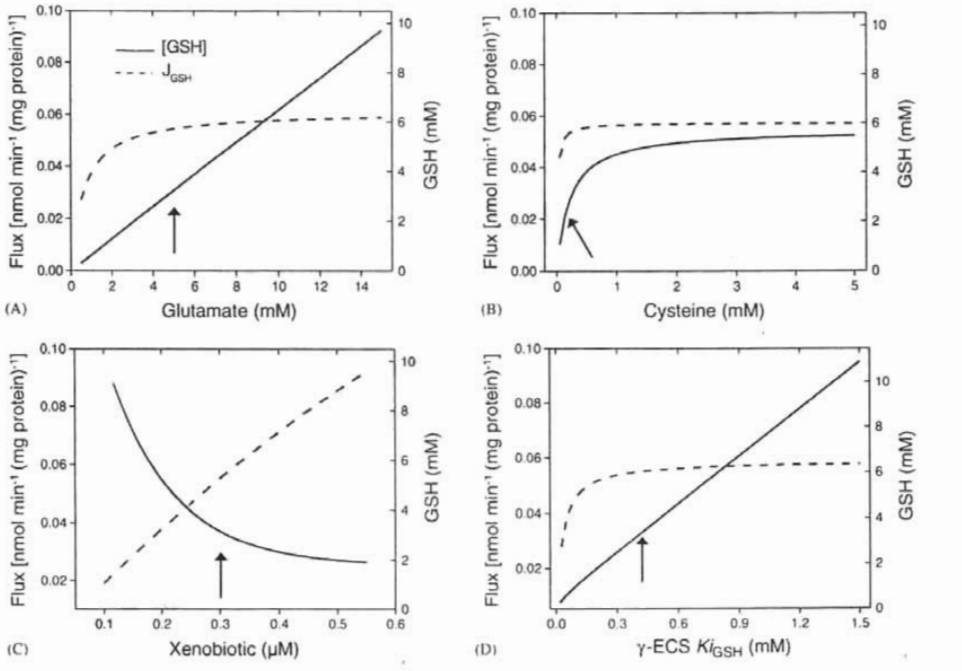


Fig. 2. Effect on flux to GSH and GSH concentration of varying the concentration of Glu (A), Cys (B), or Xe (C) and the K_i value of γ-ECS by GSH (D). The arrows indicate the physiological value of the metabolites found in plants or the K_i value used in the present kinetic model.

considered as a crucial metabolite for GSH synthesis because it is maintained roughly stable in the cell (Noctor et al., 1998), but due to the competitive nature of the feedback inhibition of γ-ECS by GSH, changes in Glu concentration may directly affect the GSH concentration.

Plants fed with an excess of sulfur develop increased Cys and GSH concentrations (Buwalda et al., 1988). The present model reproduces this response. In the range of concentrations found in plants of 0.1–1 mM (Hell and Bergmann, 1990; Noctor et al., 1998), Cys affects GSH concentration, and to a much smaller

extent J_{GSH} (Fig. 2B). This response, in which an increased GSH is associated with increase in Cys, has also been experimentally determined by several groups (Xiang and Oliver, 1998; Domínguez-Solís et al., 2001; Harada et al., 2002; Mendoza-Cózatl et al., 2002; Avilés et al., 2003). This implies that Cys synthesis from sulfate is a highly responsive pathway towards Cys variations and is able to maintain a constant or increased Cys concentration during high Cys demand. Thus, for instance under Cd^{2+} stress, which prompts a high Cys (and GSH) demand, the plasma membrane sulfate transporters and some enzymes of sulfate reduction such as ATP sulfurylase and O-acetylserine(thiol)lyase are up-regulated in plants, leading to an enhanced Cys level (Xiang and Oliver, 1998; Domínguez-Solís et al., 2001; Harada et al., 2002).

Gly is probably the amino acid with more variation in plants, particularly in plants with C3 metabolism, since it is derived from photorespiration, which shows a circadian rhythm. However, variation in Gly in the reported values of 0.1–1 mM (Noctor et al., 1997) had no effect on J_{GSH} or GSH concentration (not shown; see Supplementary data).

It is well documented for several cell lines from mammals (Habig et al., 1974; Meister, 1995), yeast (Dennda and Kula, 1986) and plants (Droog et al., 1995; Table 2), that the maximal activity of GSTs is several-fold greater than that of the enzymes responsible for GSH synthesis (Table 2). In addition, GSTs have a high affinity by GSH and at the physiological levels of GSH (1–10 mM) these enzymes are practically saturated (near-zero elasticity towards GSH). Therefore, the rate of GSTs is governed by the concentration of the electrophilic compound (Xe in this model), thus establishing a highly efficient detoxifying system. Hence, a Xe concentration that allows a physiological accumulation of GSH was selected for modeling (0.3 μM; Fig. 2C). Around this value (0.1-0.5 μM), Xe concentration affects both flux to GSH and GSH concentration.

Feedback inhibition in metabolic pathways is an efficient mechanism for metabolite homeostasis especially for those involved in reactions with large equilibrium constants (Hofmeyr and Cornish-Bowden, 1991; Cornish-Bowden and Cardenas, 2001). All described γ-ECSs, enzymes catalysing a reaction with a large K_{eq} (Table 2), show feedback inhibition by GSH, although their K_i values vary among organisms (Griffith and Mulcahy, 1999). Hence, the effect of varying K_i value was evaluated (Fig. 2D). GSH concentration was markedly affected by changing the K_i value of γ-ECS in the range tested (0.1–1.5 mM), whereas the effect on J_{GSH} was negligible.

Only K_i values below 0.2 mM have a significant effect on J_{GSH}; the meaning of this result may be physiologically irrelevant since the K_i values reported for γ-ECSs range from 0.1 to 8 mM (Griffith and Mulcahy, 1999) and above 0.2 mM the K_i value shows no effect on J_{GSH}. This finding predicts that the intracellular GSH concentration of a given organism is determined, at least in part, by the K_i value of its γ-ECS for GSH. The same conclusion may be drawn by plotting the effect of varying the K_i of γ-ECS in a supply–demand rate characteristic graph (see below for description); changing the K_i value has an effect on the GSH concentration at the different K_i values tested, whereas J_{GSH} is only affected at the lowest K_i values where the elasticity of the demand block increases, thus diminishing its control on J_{GSH} (see Supplementary data).

4.2. Control of GSH synthesis under unstressed conditions

Modeling GSH synthesis under unstressed conditions (Fig. 1, Scheme 1) shows that J_{GSH} is mainly controlled by GSTs (88%; Table 5), the GSH-consuming block, due to its lower elasticity towards GSH ($\varepsilon^{GST}_{GSH} = 0.10$) than that of the enzymes of the supply block ($\varepsilon^{\gamma\text{-ECS}}_{GSH} = -0.84$; $\varepsilon^{GS}_{GSH} = -0.91$). The $\varepsilon^{\gamma\text{-ECS}}_{GSH}$ is high, despite the saturating inhibitor concentration ($K_{iGSH} = 0.42$ mM, [GSH] = 3.4 mM at the steady state), because the GSH inhibition is of the competitive type against the substrate glutamate, which in turn is also present at a high concentration (K_{mGlu} 10.4 mM, [Glu] = 5.25 mM; Table 3). Within the supply block, γ-ECS is the controlling step because it is virtually insensitive to its immediate product ($\varepsilon^{\gamma\text{-ECS}}_{\gamma\text{-EC}} =$

Table 5
Flux control coefficients and fluxes of the glutathione and phytochelatin synthesis

Enzyme	Unstressed conditions	Cadmium exposure	
	GSH synthesis[a]	GSH synthesis[a]	Phytochelatins synthesis[b]
γ-ECS	0.11	0.58	0.60
GS	<0.01	<0.01	0.01
GSTs	0.88	0.01	−0.06
PCS	N.A.	0.40	0.44
Phytochelatins transport	N.A.	< 0.01	< 0.01
	Flux nmol min^{-1} $(mg\ protein)^{-1}$		
	GS[a]	GS	PCS[b]
	0.055	0.67	0.21

N.A. non-applicable.
[a]The flux is considered as the flux through GS. Because under unstressed conditions the pathway is linear, the values of C^J_{Ei} of each enzyme for flux through any point in the pathway are the same.
[b]The flux is considered as the flux through phytochelatin synthase. In this case there is branching after GSH and two fluxes are derived, through GSTs and PCS

-0.005; $\varepsilon^{GS}_{\gamma\text{-EC}} = 0.95$). Nevertheless, γ-ECS, the presumed rate-limiting step of the pathway (Meister, 1995; Griffith and Mulcahy, 1999), exhibits a discrete flux control (11%) on GSH synthesis. As GS flux-control is negligible (<0.01; Table 5), the connectivity theorem for GSH ($\sum \varepsilon^{Ei}_{GSH} C^J_i = 0$) reduces to $(C^J_{\gamma\text{-ECS}}/C^J_{GST}) = -(\varepsilon^{GST}_{GSH}/-\varepsilon^{\gamma ECS}_{GSH})$, which yields the same above-mentioned control profile for the supply and demand blocks.

γ-ECS is certainly a rate-limiting step ($C^J_{\gamma-ECS} = 0.99$) only whether the GSH-consuming block is not included in the model (not shown; see Supplementary data), but studying a pathway without considering the consumption (demand) of the final product is misleading. The robustness of the model was analysed by systematically changing the Michaelis constants (K_{mS}'s and K_{mP}'s of each enzyme) by a factor of 2. Neither J_{GSH} nor the C^J_{GSTs} were significantly affected (>10%) by modifying the K_m values, whereas the $C^J_{\gamma\text{-ECS}}$ varied between 0.05 and 0.19 only when the K_{mGlu} or the K_{iGSH} were altered.

The feedback inhibition of γ-ECS by GSH reveals efficient transfer information from the ending to the initial pathway segment (Cornish-Bowden and Cardenas, 2001). This allows that GSH synthesis in unstressed conditions may also be modeled using irreversible equations for γ-ECS and GS without major alterations in the control structure of the pathway (<10%; see Supplementary data).

To further support the initial results on flux control reached by the GSH synthesis modeling, the pathway was divided in two blocks (supply and demand) and their rate characteristics (Hofmeyr, 1995) in response to fixed concentrations of GSH were obtained. Plotting the local steady-state flux of both blocks, in logarithmic scale, against the GSH concentration also in logarithmic scale, a control analysis according to the supply–demand theory developed by Hofmeyr and Cornish-Bowden (2000) was made (Fig. 3A). The intersection of supply and demand rates denotes the steady-state flux (J) and GSH concentration (arrow in Fig. 3A). From the slope at the steady-state point, the elasticity for each block is determined. Following the summation and connectivity theorems (Eqs. 6(6) and (5), respectively),

$$C^J_{supply} + C^J_{demand} = 1, \tag{6}$$

$$C^J_{supply}\varepsilon^{supply}_{GSH} + C^J_{demand}\varepsilon^{demand}_{GSH} = 0 \tag{7}$$

values for C^J_{supply} and C^J_{demand} are derived (0.1 and 0.9, respectively), which are in good agreement with those attained with the algorithm of *GEPASI*. As GS shows no control on the pathway, then C^J_{supply} becomes $C^J_{\gamma\text{-ECS}}$.

From the kinetic parameters of GSTs (Table 2), one can realize that these enzymes are physiologically saturated by GSH (Fig. 3A) and thus their rate is predominantly regulated by the Xe concentration. If the

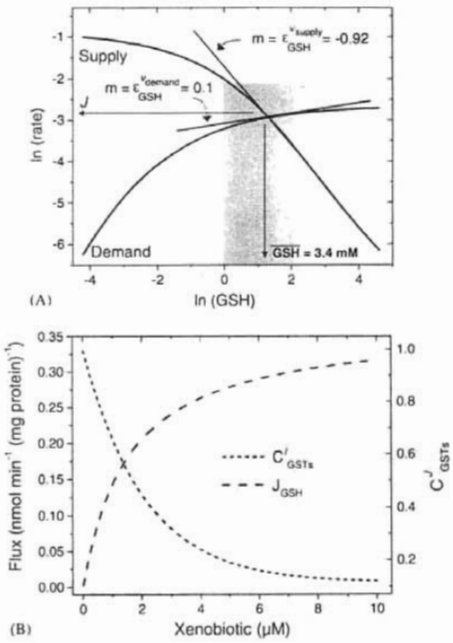


Fig. 3. (A) Supply–demand analysis of the GSH biosynthesis pathway. The rate characteristics of each block were obtained by fixing GSH at different concentrations and using the software Microcal ORIGIN 5.0 (*Microsoft*). The slope at the intercept (steady state) of the rates of the supply and demand block represents the elasticity of each block toward GSH. Elasticities were used to obtain the flux control coefficient of each block using the connectivity theorem. (B) Effect on flux to GSH and $C^J_{\gamma\text{-ECS}}$ (supply block) of increasing the Xe concentration and hence the activity of the demand block.

increase in GSTs activity, induced by herbicide or heavy metal exposure, surpasses the rate of GSH synthesis, then it is expected that part of the flux-control transfers to the supply block. Hence, by increasing the Xe concentration and hence the GSTs activity, the C^J_{demand} (GSTs) diminishes and flux control is transferred to the supply block (γ-ECS; Fig. 3B). At low GSH demand, the GSH-consuming block exerts more control whereas, at high demand, the supply block mainly controls GSH synthesis.

The notion that γ-ECS is the rate-limiting step of GSH synthesis is based on the physiological GSH feedback inhibition by GSH (Meister, 1995; Griffith and Mulcahy, 1999). This view has been reinforced by the finding that over-expression of *E. coli* γ-ECS in plants under unstressed conditions promotes an increase in GSH concentration (Noctor et al., 1997, 1998; Zhu et al., 1999b). It is worth noting that the concept of rate-limiting step has been used to describe an enzyme that

has control, indistinctly, on the rate of synthesis of a given end product metabolite and/or on its steady-state concentration. This empirical statement may or may not be accurate.

Control analysis of the modeled pathway shows that γ-ECS has an elevated $C^{GSH}_{\gamma\text{-ECS}}$ (Table 6), which means that an increase in the γ-ECS activity would proportionally increase the GSH concentration (Fig. 4A). The demand block has also a high and identical but negative C^{GSH}_{GST}, which is accompanied by a high C^{J}_{GST}; in this case, inhibition of GSTs (diminution in Xe concentration, Fig. 2C) would also increase GSH concentration. Furthermore, modeling shows that an increased GSH concentration can also be achieved by only altering the K_i value of γ-ECS for GSH (Fig. 4); keeping constants the rates of GSH supply (γ-ECS activity = 1 in Fig. 4) and demand, GSH was 3.4 (Fig. 4A) or 14 mM (Fig. 4B), depending on the K_i value. These model predictions provide a mechanistic explanation for the increased GSH content achieved when overexpressing *E. coli* γ-ECS in plants (Noctor et al., 1997, 1998; Zhu et al., 1999b): K_i of *E. coli* γ-ECS for GSH ≈ 2 mM, a higher value than that of plant γ-ECS. Note that in all these manipulations, flux to GSH remains unaltered.

Table 6
Concentration control coefficients of GSH and phytochelatins synthesis pathway

Enzyme	Unstressed conditions	Cadmium exposure	
	GSH	GSH	Phytochelatins
γ-ECS	1.05	0.68	0.76
GS	<0.01	0.01	0.01
GSTs	−1.05	−0.07	−0.07
PCS	N.A.	−0.63	0.56
Phytochelatins transport	N.A.	<0.01	−1.2

N.A. non-applicable.

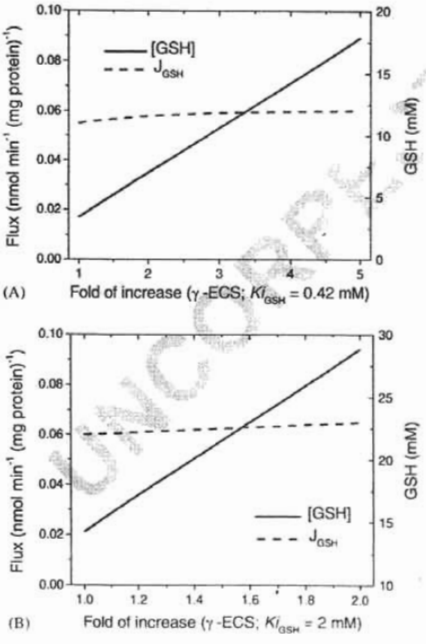


Fig. 4. Simulation of increasing the γ-ECS activity with different K_{iGSH} values: (A) K_{iGSH} = 0.42 mM, and (B) K_{iGSH} = 2 mM.

Variation in the Glu or Cys concentration (Fig. 2A and B) does not affect the flux control distribution (Table 5); the C^{J}_{GST} ranges from 0.88 to 0.95. Major changes in control distribution are achieved when the metabolites diminish below their respective physiological concentrations. Changes in Gly concentration in the range 0.1–1 mM have no effect on the control structure (see Supplementary data).

4.3. GSH synthesis under cadmium exposure

When plants are exposed to Cd^{2+} phytochelatin synthesis, another GSH-consuming branch is activated thus increasing the GSH demand (Fig. 1, Schemes 2 and 3). One well-known cellular response to Cd^{2+} stress is the up-regulated transcription of γ-ECS and GS genes leading to 3–7-fold higher mRNA levels (Xiang and Oliver, 1998; reviewed by Mendoza-Cózatl et al., 2005). Less known is the effective increase in enzyme activity. Analysis of literature data reveals an average of 2.5 times increase in both enzyme activities (Tsuji et al., 2003; Martínez-Oliver J, Mendoza-Cózatl D, Moreno-Sánchez R, unpublished results). Therefore, such an increase in enzyme activity under Cd^{2+} stress was incorporated in the model (Table 2). In this branched pathway, control analysis is focused on flux to GSH J_{GSH} (through GS) and on the bio-technologically orientated flux to phytochelatins J_{pcs} (through PCS) (Table 5).

Modeling shows that cadmium up to a concentration of 5 μM increases both fluxes, J_{GSH} and J_{pcs} (Fig. 5A, Table 5 bottom); higher concentrations had a discrete effect. Cadmium also diminishes the GSH pool, but increases the phytochelatins content (Fig. 5B). This modeled effect of Cd^{2+} on GSH synthesis may appear somewhat difficult to explain. For instance, plants exposed to 50 μM $CdCl_2$ show an increase, not decrease, in the GSH concentration (Dominguez-Solis et al., 2001). However, higher cadmium concentrations certainly induce a decrease in the GSH content and an increase in phytochelatins concentration (Meuwly and

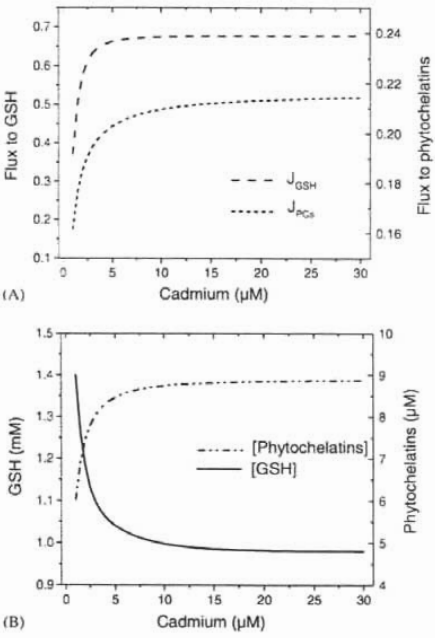


Fig. 5. Effect of cadmium increase on: (A) flux to GSH (dashed line) and flux to phytochelatins (short-dashed line), and (B) GSH concentration (straight line) and phytochelatins concentration (dash-dotted line).

Rauser, 1992). Cd^{2+} was included in the model as free cytosolic divalent cation; however, in an in vivo situation, other molecules such as phosphate, pyrophosphate, and organic acids (citrate, malate) may also bind Cd^{2+}. Further pathway modeling under Cd^{2+} stress was made using a cadmium concentration of 10 μM, which still maintains a GSH concentration above the lower physiological reported value of 1 mM (Noctor and Foyer, 1998; Table 4).

Flux control is redistributed as expected from an increased GSH demand (Table 5). Due to their low elasticity towards their respective substrate, Cys ($\varepsilon^{\gamma\text{-ECS}}_{Cys} = 0.17$) and GS_2Cd ($\varepsilon^{PCS}_{GS2Cd} = 0.06$), γ-ECS and PCS now control J_{GSH} and J_{pcs}; in addition, γ-ECS shows the lowest elasticity for GSH ($\varepsilon^{\gamma\text{-ECS}}_{GSH} = -0.61$). γ-ECS and PCS also control the GSH concentration whereas phytochelatins concentration is controlled by γ-ECS, PCS and significantly by the vacuolar transporter (Table 6). The robustness of the Cd^{2+} exposure model was also assessed by changing the Michaelis constants by a factor of 2. The control structure of the pathway remains unaltered by these small changes, although the control of the flux to GSH and PCs by PCS varies between 0.37 and 0.47 when its K_{mGSH} is modified.

It has been suggested that γ-ECS plays an important protective role under stress conditions (i.e. oxidative, heat or heavy metal stress) (Xiang and Oliver, 1998; Dormer et al., 2002). Over-expression of *E. coli* γ-ECS promotes an increase in the cadmium resistance and accumulation capacity in *B. juncea* (Zhu et al., 1999b). However, high over-expression of the same enzyme in tobacco leads to oxidative stress due to accumulation of the oxidized form of γ-EC, ESSE (Creissen et al., 1999). The model (Fig. 6A and B) can reproduce these experimental findings. As γ-ECS has significant control on GSH and phytochelatins synthesis under Cd^{2+} stress (Table 5), then by increasing its activity, enhancement in J_{GSH} and J_{pcs} is achieved as well as in GSH and phytochelatins concentrations. Although this result is expected to increase the cadmium resistance and accumulation capacity, a negative side effect is the high accumulation of γ-EC over physiological levels (Table 4; compare γ-ECS 5 × versus *no changes* in the cadmium exposure columns). This accumulation may induce additional oxidative stress along with that promoted by cadmium itself.

Considering that the PCS gene has been cloned, and that phytochelatins are directly related with Cd^{2+} inactivation (for a review, see Mendoza-Cózatl et al., 2005), then an obvious experiment is to simulate over-expression of PCS to increase the cadmium resistance (Fig. 6C and D). The model predicts that PCS over-expression promotes elevation of the phytochelatins content, although the GSH level severely diminishes (see also Table 4; PCS 5 ×). Inability to maintain GSH levels under Cd^{2+} exposure may lead to oxidative stress and Cd^{2+} hypersensitivity, as demonstrated experimentally by over-expressing PCS in *A. thaliana* (Lee et al., 2003). In addition, the increase in PCS activity, although promotes a higher J_{PCs}, shows little effect on J_{GSH} (Fig. 6C). Therefore, to achieve the proposed biotechnological orientated goal of an enhanced Cd^{2+} accumulation capacity, flux to phytochelatins should be increased without compromising the GSH level required for oxidative stress processing.

The *Universal Method* of Kacser and Acerenza (1993) addresses the problem of inducing an increase in a given flux, and hence in a desired end-product metabolite, without affecting the concentrations of all other intermediaries and branched fluxes. To achieve such a goal the method proposes to increase the activity of all enzymes involved in both the input to the *crossroad* metabolite (GSH) and the output towards the desired metabolite (phytochelatins). Thus, for the Cd^{2+} stress model, the flux through GS of 0.67 (Table 5), or 100%, splits into four fluxes with values of 0.21 (PCS), or 31.3%, 0.04 (GSTs), 0.21 (GS_2-Cd), and 0.21 (γ-EC release from GSH during phytochelatins formation; Fig. 2). Then, to increase flux through PCS without affecting both the GSH concentration and the flux through GSTs,

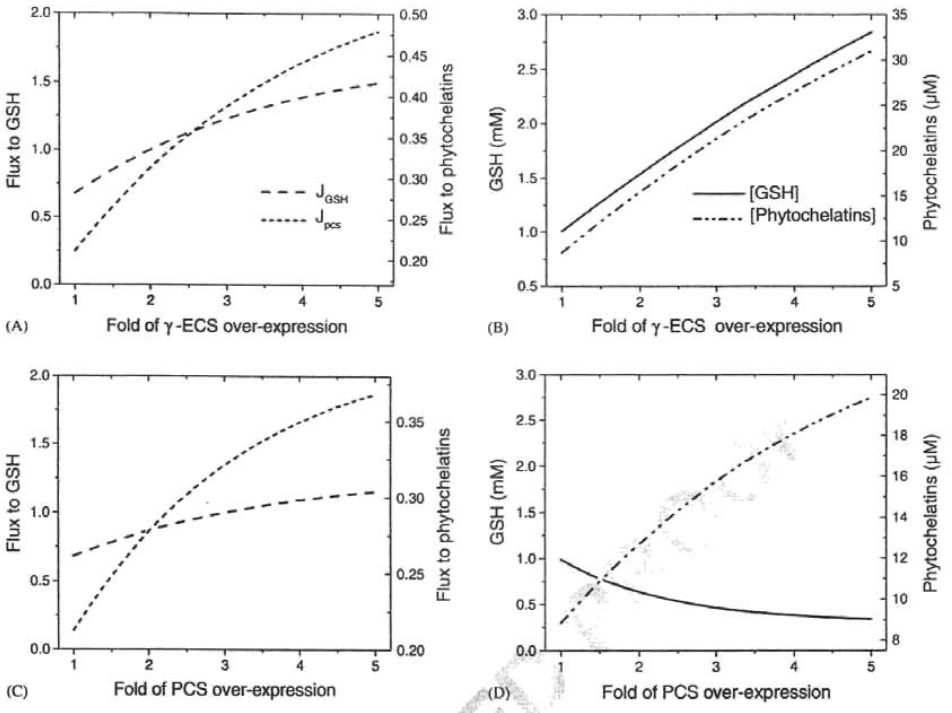


Fig. 6. Effect of increasing γ-ECS activity on: (A) flux to GSH (dashed line) and flux to phytochelatins (short-dashed line), (B) GSH concentration (straight line) and phytochelatins concentration (dash-dotted line). Effect of increasing PCS activity on (C) flux to GSH (dashed line) and flux to phytochelatins (short-dashed line), and (D) GSH concentration (straight line) and phytochelatins concentration (dash-dotted line).

the *Universal Method* establishes that any given degree of PCS over-expression should be accompanied by a proportional 0.31 increase (respect to the PCS increase) in γ-ECS and GS over-expression. However, such differential over-expression of several enzymes may be experimentally difficult to accomplish.

Modeling shows that γ-ECS over-expression is an effective protocol to increase the GSH content, although with detrimental side effects (accumulation of ESSE). By simulating a more realistic simultaneous over-expression to the same degree of the relevant enzymes in the supply and demand (Table 4, γ-ECS + PCS 2.5 ×; Fig. 7A and B), as an useful approximation of the *Universal Method*, it is possible to effectively increase J_{pcs} as well as J_{GSH} by 3 and 2.5 times, respectively, without a significant accumulation of γ-EC (Table 4). Moreover, the GSH pool is not depleted and phytochelatins concentration increased by 4.5 times. Organisms with these characteristics may develop a Cd^{2+} hyper-accumulating phenotype.

Inhibiting other GSH-consuming enzymes (represented by GSTs in this model) may also enhance the levels of GSH and phytochelatins and their respective fluxes. This possibility seems less feasible because GSTs are involved in detoxification of compounds derived from oxidative stress such as 4-hydroxyalkenals and base propanols (Marrs, 1996). However, by inhibiting GSTs to an extent that does not compromise the cell ability to detoxify intracellular toxic compounds, it may also be promoted an increase in J_{GSH} and J_{pcs} with a concomitant increase in GSH and phytochelatins concentrations.

Modeling of GSH biosynthesis was made assuming that ATP was saturating (see Appendix A). This assumption may have some validity under control, unstressed conditions, but definitively can be challenged under stress conditions that may affect the ATP-generating pathways (Vallee and Ulmer, 1972; Devars et al., 1998). The affinity of the tobacco γ-ECS and GS for ATP is not available; however, most of the characterized enzymes from bacteria, yeast and protists show K_m values for ATP below 1 mM (Griffith and Mulcahy, 1999; Meierjohann et al., 2002). The ATP dependence of J_{GSH} and GSH concentration may be

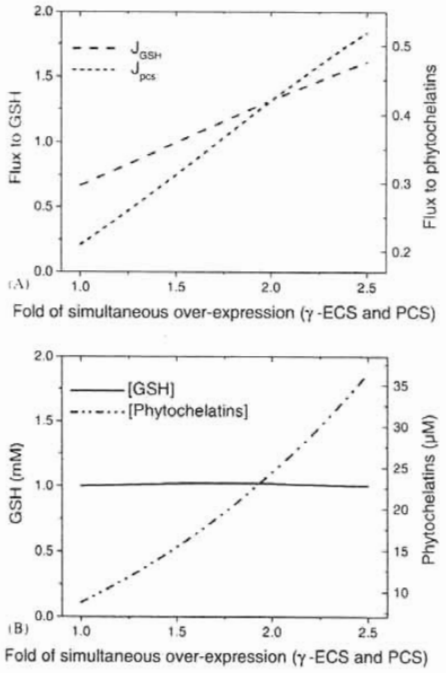


Fig. 7. Effect of simultaneous increase in γ-ECS and PCS activity on: (A) flux to GSH (dashed line) and flux to phytochelatins (short-dashed line), and (B) GSH concentration, (straight line) and phytochelatins concentration, (dashed-dotted line).

evaluated using an additional model based on the kinetic properties of yeast, including the K_m for ATP (see Supplementary data). The yeast model predicts that above 2 mM ATP, the GSH level is roughly stable, but below 2 mM, ATP steeply diminishes. Therefore, a similar response to ATP variation may be expected for the plant system.

5. Discussion

Modeling metabolic pathways is a powerful tool in the sense that it simulates properties of a given pathway that may not be deduced by simple inspection of the kinetic parameters of the enzymes, and which may be relevant to understand the biochemical mechanisms involved in the regulation of a pathway. Modeling involves the quantitative consideration of the multitude of reported data and interactions that are typical of a biological system, allowing for an integrative view of sometimes disperse data, discarding irrelevant facts (Heinrich et al., 1977). Although models are simplifications of the complex reality, they are useful for the deduction of essential relations. In addition, modeling may help to design experimental strategies to test the different hypotheses on the pathway control and may also help to detect incompatibilities on the kinetic parameters of the different participating enzymes, prompting experimental revision of the most critical enzyme uncertainties.

In this work, we have constructed a kinetic model of the GSH and phytochelatins synthesis under control and Cd^{2+}-stressed conditions to understand how this pathway is controlled, and to identify which the targets for genetic manipulation may be to increasing the heavy metal resistance-accumulation ability of the organisms. This is the first attempt to determine the control structure of the GSH–phytochelatins synthesizing pathway and to explain why the efforts to increase the GSH and phytochelatins content by over-expressing some enzymes involved in this pathway have achieved limited success.

The enzymes responsible of GSH synthesis from its constituent amino acids (γ-ECS and GS; Fig. 1, Scheme 1) catalyse reactions with large equilibrium constants (>1000). Then under low-GSH demand this block of enzymes has to receive information from the end of the pathway, other than simple product accumulation and hence inhibition, to avoid high accumulation of the intermediaries (γ-EC and GSH) and to achieve a stable steady-state flux (Cornish-Bowden and Cardenas, 2001). This information transfer is provided by the feedback inhibition of γ-ECS by GSH (Meister, 1995). The existence of such mechanism has led to the proposal that γ-ECS is the rate limiting step of GSH synthesis (Meister, 1995; Noctor et al., 1998; Zhu et al, 1999a, b). In agreement with this notion, pathway modeling shows that when GSH-consuming enzymes are not included, γ-ECS is indeed the rate-limiting step ($C^J_{\gamma\text{-ECS}} = 0.99$) of the pathway.

However, it seems more accurate to analyse GSH synthesis pathway with the product consumption reactions included, because it has been demonstrated that significant flux control may reside outside the synthetic block of enzymes, i.e. in the consuming block of enzymes (Rodriguez-Enriquez et al., 2000; Hofmeyr and Cornish-Bowden, 2000; Koebmann et al., 2002). With this consideration taking into account, modeling shows that under low demand, the demand block mainly controls J_{GSH}, whereas at high demand, the supply block exerts the major flux control on GSH synthesis (Fig. 3A and B; Table 4).

On the other hand, this model emphasizes that control on the rate of GSH synthesis is not directly related with control on the GSH concentration under unstressed conditions. GSH concentration is homeostatically maintained by γ-ECS, the enzyme that senses the information transfer from the end of the pathway (Hofmeyr and Cornish-Bowden, 2000; Cornish-Bowden

and Cardenas, 2001). It is important to recall that GSH concentration is not solely controlled by γ-ECS. In a supply–demand system, the control over the concentration of the metabolite that links supply and demand is determined by the sum of the elasticities of supply and demand. Under unstressed conditions, the GSH demand elasticity was near zero (so that demand has almost full control over the flux; Table 5). Thus, the GSH concentration control is predominantly determined by the supply elasticity, which was near-one. In consequence, while supply and demand have equal but opposite control over the concentration, the absolute value of their control coefficients is in this case mainly determined by the supply elasticity: the larger this elasticity, the smaller the concentration control coefficient. It is therefore in this sense that the supply (γ-ECS) regulates the degree of homeostasis in the concentration of the linking metabolite (GSH) and exerts little flux control.

Cadmium exposure represents a situation of high-GSH demand (see the increase in flux in Table 5 bottom). Consequently, the control of the pathway is redistributed and both blocks (supply and demand) share the control of GSH synthesis and GSH concentration (Tables 5 and 6). It is worth to note that cadmium exposure induces oxidative stress and, depending on the concentration, it may alter the redox state of the cell. In such case, GPx and GR may play a crucial role in the GSH homeostasis. In addition, because GR reduces GSSG at expenses of NADPH, forming part of a moiety-conserved cycle, additional control properties to the pathway may be conferred by GR (see Hofmeyr et al. (1986) for MCA of moiety-conserved cycles). Unfortunately, few works, if any, have determined systematically the effect of cadmium on the cellular redox state and on activity of GR and GPx (there are many works dealing with gene expression but this may not directly reflect the effect on enzyme activity).

Experimental data regarding how the GSH and phytochelatins synthesis pathway may respond to an increased demand of GSH are also scarce. Furthermore, how an increased activity of an enzyme may compromise the pool of a metabolite used for several reactions (i.e. GSH; Kim et al., 2003), inducing secondary undesirable effects (accumulation of reactive intermediaries such as oxidized γ-EC; Creissen et al., 1999) has also been poorly explored. Pathway modeling shows that the most effective way to increase the GSH content is to enhance the γ-ECS activity (Tables 4; Fig. 6A and B), although such manipulation brings about little effect on the phytochelatins concentration. This is because GSH can be used in several reactions besides phytochelatins synthesis and because PCS is not by far a highly efficient enzyme ($V = 3.2\,\text{nmol}\,\text{min}^{-1}\ (\text{mg protein})^{-1}$; $K_{mGSH} > 10\,\text{mM}$) (Table 2).

PCS has been proposed as a genetic target for improving the Cd^{2+} resistance because its product is directly related to Cd^{2+} sequestration and transport (Vatamaniuk et al., 2002). The model shows that when only PCS is over-expressed, there is a depletion of the GSH pool (Fig. 6C and D). Inability to maintain GSH levels under Cd^{2+} exposure may lead to oxidative stress and Cd^{2+} hypersensitivity. This last phenotype is indeed observed when PCS is over-expressed in *A. thaliana* (Lee et al., 2003). However, *E. coli* PCS expression in *S. cerevisiae*, which does not have the PCS gene, induces a higher Cd^{2+} resistance (Clemens et al., 1999). Then, it should be taken into consideration, when a high PCS over-expression is achieved, that this manipulation may have a detrimental effect whether GSH is lowered below a critical value.

In consequence, an approximation of the *Universal Method* was applied to the problem of increasing the phytochelatins concentration without compromising the GSH content. The high control coefficients of γ-ECS and PCS on flux to GSH and phytochelatins and on the GSH and phytochelatins concentrations (Tables 5 and 6) make these enzymes potential genetic targets for enhancing the GSH and phytochelatins levels. Indeed, the simultaneous increase in the γ-ECS and PCS activities results in the most successful simulation to achieve an increased phytochelatins concentration without depleting the GSH pool (Fig. 7A and B).

It should be noted that Cys synthesis is a very efficient pathway able to respond to an increased Cys demand induced by either Cd^{2+} stress or over-expression of Cys-consuming enzymes, i.e. γ-ECS (Noctor et al., 1998; Creissen et al., 1999; Dominguez-Solís et al., 2001). Analysis of the literature data about the kinetics properties of the enzymes involved in Cys synthesis (Mendoza-Cózatl et al., 2005) shows that these enzymes are more efficient that γ-ECS (as judged by the V/K_m ratio). This suggests that, as in the case of GSH synthesis, Cys synthesis may also be mainly controlled by the Cys-demand block (GSH synthesis, protein synthesis).

6. Concluding remarks

1. The rate of GSH synthesis in unstressed conditions is mainly controlled by the consuming block of enzymes (demand).
2. While γ-ECS is the rate-limiting step of GSH synthesis when studied on its own, it is not rate-limiting when the GSH-consuming reactions (demand) are taken into account. The role of feedback inhibition of γ-ECS by GSH is to allow the sensing of variations in GSH concentration, thereby determin-

ing the homeostatic maintenance of GSH. However, it does not determine the flux control of the pathway.

3. A clear distinction should be made regarding which steps control the rate synthesis of GSH and which steps control the GSH concentration; depending on the cellular status, the same set of enzymes may, or may not, exert control on both pathway properties.
4. Under Cd^{2+} stress, and an increased GSH demand, both synthesis and demand blocks share the flux control to GSH and phytochelatins as well the concentration control of both metabolites.
5. The manipulation of the GSH and phytochelatins synthesis pathway to enhance Cd^{2+} resistance and accumulation should proceed by increasing simultaneously expression, and activity, of γ-ECS and PCS. Over-expression of these two enzymes may bring about an elevation in both flux and concentration of relevant metabolites. This sort of genetic manipulation may confer the desired characteristics in a much greater extent than those reached by manipulating only one enzyme and should prevent the accumulation of toxic intermediaries such as ESSE.

Acknowledgements

The authors thank Prof. J.H.S. Hofmeyr for his thorough revision of the manuscript and critical observations. The present work was partially supported by grants No. 43811-Q from CONACyT-México and PAPIIT-IN215705 from DGAPA-UNAM.

Appendix A

A.1. γ-ECS and GS

γ-ECS and GS are strictly ter-reactant enzymes, which form a peptide bond at expenses of the ATP hydrolysis (Meister, 1995). The reported K_{mATP} values for both enzymes are in the majority of the organisms below 1 mM (Griffith and Mulcahy, 1999; Meierjohann et al., 2002). Since cytosolic ATP is usually above 0.7 mM in plants (Geingenberger et al., 2001; van Dongen et al., 2003) and several cellular processes are involved in maintaining its concentration roughly constant, ATP is expected to play a minor role in determining the rate of both reactions; therefore, it was not included in the rate equations. Thus, reversible bi-reactant equations were used for these two enzymes.

On the other hand, kinetic parameters of the reversibility of γ-ECS and GS under physiological conditions are not available very likely because the large K_{eq} of their reactions prevents such possibility. However, significant changes in the control structure have been found by introducing reversibility in all pathway reactions, even in those with very large K_{eq} (i.e. pyruvate kinase, $K_{eq} = 10^4$; see Cornish-Bowden and Cardenas (2001) for reversibility in kinetic models). The use of reversible equations, although more accurate than irreversible equations, brings about a major experimental problem: how to include kinetic parameters that do not exist in the literature and that, due to experimental difficulty, would be unreliable. This is particularly troublesome for the maximal rate of the reverse reaction (V_r) and, to a lesser extent, for the enzyme affinity for the product (K_P). An irreversible rate equation could still be a good approximation for reactions with large K_{eq} when the enzyme K_P value is much higher than the physiological product concentration (or when V_r is extremely low in comparison to V_f). This would avoid incorporation of reversible equations with guessed values in pathway modeling.

By introducing the K_{eq} value of the overall reaction, V_r is discarded. γ-ECS and GS have the same K_{eq} since both catalyse the same reaction regarding a peptide bond formation at expenses of ATP hydrolysis. $\Delta G^{\circ\prime}$ for a peptide bond formation is +2.2 kcal/mol whereas the $\Delta G^{\circ\prime}$ of ATP hydrolysis is −7.3 kcal/mol (Lehninger et al., 2000); the difference (−5.1 kcal/mol) may be used to calculate the K_{eq} of the overall reaction, $K_{eq} = 5597$ (Table 2). This value is an underestimate because the ΔG of ATP hydrolysis under many physiological conditions may be higher (> -9 kcal/mol) than the standard value.

The K_P values for γ-EC and GSH used in the pathway modeling were estimated as follows. γ-ECS is able to perform the synthesis of γ-EC in the presence of 1 mM Cys, 20 mM Glu and 4 mM ATP with linear rates for up to 60 min (Hell and Bergmann, 1990). This means that accumulation of γ-EC up to a concentration of 0.5 mM has no effect on the initial rate of the enzymes. With the kinetic parameters described in Table 2, the γ-ECS activity was simulated against different K_P values (Fig. 8A; note that the x-axis is plotted in log-scale to visualize the effect of large changes in the K_P value). The K_P values that do not affect the rate of γ-ECS during the accumulation of 0.5 mM of its product (γ-EC) should be greater than 0.3 mM. The same treatment was done for GS. The initial rate of GSH synthesis with 1 mM of γ-EC and Gly was not affected by the accumulation of 0.075 mM GSH (Hell and Bergmann, 1988). This means that the K_P value for GSH of GS has to be above 0.03 mM (Fig. 8B). Furthermore, the effect of simultaneous variation in the K_P values of both enzymes on the flux to GSH in unstressed conditions was simulated (Fig. 8C). In any case, K_P should be lower than 0.01 mM to significantly alter the flux to GSH. Therefore, the K_P values used in the modeling of GSH biosynthesis were 0.3 mM for γ-ECS and 0.03 for GS.

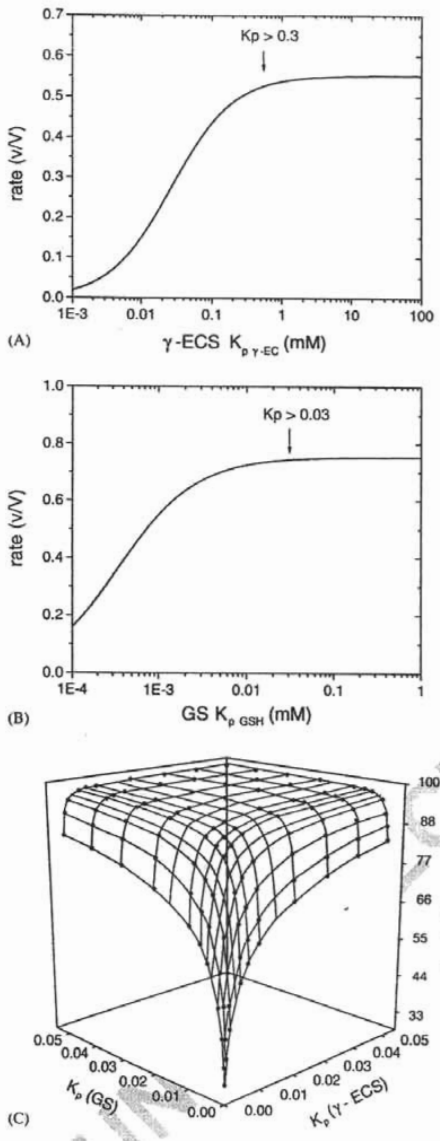


Fig. 8. (A) Effect of $K_{p\gamma\text{-}EC}$ value of γ-ECS on the rate (normalized) of γ-EC synthesis under initial rate conditions. The *x*-axis (K_p) is plotted in log-scale to visualize the effect of large changes in the K_p value. Kinetic parameters are listed in Table 2; substrate and product concentrations were Cys = 1 mM, Glu = 20 mM and γ-EC 0.5 mM. (B) Effect of K_{pGSH} value of GS on the rate (normalized) of GSH synthesis under initial rate conditions. Substrate and product concentrations were γ-EC = 1 mM, Gly = 1 mM and GSH 0.075 mM. (C) Effect of the K_i value of γ-ECS and GS on the flux to GSH synthesis. 100% represents 0.055 nmol min^{-1} (mg protein)$^{-1}$.

A.2. PCS kinetics

The reversibility of PCS is much more complicated if not impossible to measure or estimate. PCS catalyses the synthesis of phytochelatins (pc) according to the following equation:

$$pc_n + pc_m \Rightarrow pc_{n+1} + pc_{m-1} \quad (8)$$

When PCS is incubated with GSH and Cd^{2+}, the first products are the smallest phytochelatin (pc_2) and Gly (for a detailed revision of PCS kinetics, see Rea et al., 2004). When PCS is incubated only with pc_2 and Cd^{2+}, the products are pc_3 and GSH, not the expected GSH and γ-EC (Grill et al., 1989). This characteristic makes the PCS reaction irreversible. Phytochelatins are not accumulated in the cytosol to infinite values because the vacuolar ABC-type transporter internalizes phytochelatins into the vacuole efficiently; comparison of the kinetic properties of the vacuolar transporter and PCS clearly illustrates this point (Table 2). Thus, the length of the prevalent phytochelatins (pcs_n where $n = 2$–11) in the cytosol is determined by the efficiency of their compartmentation; the vacuolar transporter may show higher affinity for a particular phytochelatin. Therefore, PCS reaction in the present model was considered as an irreversible reaction.

Appendix B. Supplementary Data

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.jtbi.2005.07.003

References

Aviles, C., Loza-Tavera, H., Terry, N., Moreno-Sánchez, R., 2003. Mercury pretreatment selects an enhanced cadmium-accumulating phenotype in *Euglena gracilis*. Arch. Microbiol. 180, 1–10.

Bakker, B.M., Michels, P.A.M., Opperdoes, F.R., Westerhoff, H.V., 1997. Glycolysis in bloodstream form *Trypanosoma brucei* can be understood in terms of the kinetics of the glycolytic enzymes. J. Biol. Chem. 272, 3207–3215.

Bakker, B.M., Michels, P.A.M., Opperdoes, F.R., Westerhoff, H.V., 1999. What controls glycolysis in bloodstream form *Trypanosoma brucei*? J. Biol. Chem. 274, 14551–14559.

Buwalda, F., de Kok, L.J., Stulen, I., Kuiper, P.J.C., 1988. Cysteine, γ-glutamyl-cysteine and glutathione contents of spinach leaves as affected by darkness and application of excess sulfur. Physiol. Plant. 74, 663–668.

Chassagnole, C., Fell, D.A., Rais, B., Kudla, B., Mazat, J.P., 2001. Control of the threonine-synthesis pathway in *E. coli*: a theoretical and experimental approach. Biochem. J. 356, 433–444.

Clemens, S., Kim, E.J., Neumann, D., Schroeder, J., 1999. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J. 18, 3325–3333.

Cornish-Bowden, A., Cardenas, M.L., 2001. Information transfer in metabolic pathways. Eur. J. Biochem. 268, 6616–6624.

Creissen, G., Firmin, J., Fryer, M., Kular, B., Leyland, N., Reynolds, H., Pastori, G., Wellburn, F., Baker, N., Wellburn, A., Mullineaux, P., 1999. Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell 11, 1277–1291.

Dennda, G., Kula, M.R., 1986. Purification and evaluation of the glutathione-synthesizing enzymes from *Candida boidinni* for cell-free synthesis of glutathione. J. Biotechnol. 4, 143–158.

DeRidder, B.P., Dixon, D.P., Beussman, D.J., Edwards, R., Goldsbrough, P.B., 2002. Induction of glutathione S-transferases in Arabidopsis by herbicide safeners. Plant Physiol. 130, 1497–1505.

Devars, S., Hernández, R., Moreno-Sánchez, R., 1998. Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by preexposure to mercury or cadmium. Arch. Environ. Contam. Toxicol. 34, 128–135.

Dhankher, O.P., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A., Meagher, R.B., 2002. Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and γ-glutamylcysteine synthetase expression. Nature Biotechnol. 20, 1140–1145.

Dominguez-Solis, J.R., Gutiérrez-Alcalá, G., Romero, L.C., Gotor, C., 2001. The cytosolic O-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. J. Biol. Chem. 276, 9297–9302.

Dormer, U.H., Westwater, J., Stephen, D.W.S., Jamieson, D.J., 2002. Oxidant regulation of the *Saccharomyces cerevisiae GSH1* gene. Biochim. Biophys. Acta 1576, 23–29.

Droog, F.N.J., Hooykaas, P.J.J., van der Zaal, B.J., 1995. 2,4-dichlorophenoxyacetic acid related chloronated compounds inhibit two auxin-regulated type III tobacco glutathione S-transferases. Plant Physiol. 107, 1139–1146.

Fell, D., 1997. Understanding the Control of Metabolism. 1st ed. Portland Press, London 287 pp. ISBN 85578 047 XISSN 1353 6516.

Geingenberger, P., Stamme, C., Tiaden, J., Schulz, A., Quick, P.W., Betsche, T., Kersting, H.J., Neuhaus, H.E., 2001. Tuber physiology and properties of starch from tubers of transgenic potato plants with altered plastidic adenylate transporter activity. Plant Physiol. 125, 1667–1678.

Gekeler, W., Grill, E., Winnacker, E.L., Zenk, M.H., 1988. Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch. Microbiol. 150, 197–202.

Grant, C.M., MacIver, F.H., Dawes, I.W., 1997. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide γ-glutamylcysteine. Mol. Biol. Cell 8, 1699–1707.

Griffith, O.W., Mulcahy, T., 1999. The enzymes of glutathione synthesis: γ-glutamylcysteine synthetase. Adv. Enzymol. Relat. Areas Mol. Biol. 73, 209–267.

Grill, E., Löffler, S., Winnacker, E.L., Zenk, M.H., 1989. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc. Natl Acad. Sci. USA 86, 6838–6842.

Habig, W., Pabst, M.J., Jacoby, W.B., 1974. Glutathione S-transferases. J. Biol. Chem. 249, 7130–7139.

Harada, E., Yamaguchi, Y., Koizumi, N., Hiroshi, S., 2002. Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in *Arabidopsis*. J. Plant Physiol. 159, 445–448.

Harms, K., von Ballmos, F., Brunold, C., Hofgen, R., Hesse, H., 2002. Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione. Plant J. 22, 335–343.

Hatzfeld, Y., Cathala, N., Grignon, C., Davidian, J.C., 1998. Effect of ATP sulfurylase overexpression in bright yellow 2 tobacco cells. Plant Physiol. 116, 1307–1313.

Heinrich, R., Rapoport, S.M., Rapoport, T.A., 1977. Metabolic regulation and mathematical models. Prog. Biophys. Mol. Biol. 32, 1–82.

Hell, R., Bergmann, L., 1988. Glutathione synthetase in tobacco suspension cultures catalytic properties and localization. Physiol. Plant. 72, 70–76.

Hell, R., Bergmann, L., 1990. γ-glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta 180, 603–612.

Hofmeyr, J.H.S., 1995. Metabolic regulation: a control analytic perspective. J. Bioenerg. Biomembr. 27, 479–490.

Hofmeyr, J.H.S., Cornish-Bowden, A., 1991. Quantitative assessment of regulation in metabolic systems. Eur. J. Biochem. 200, 223–236.

Hofmeyr, J.H.S., Cornish-Bowden, A., 2000. Regulating the cellular economy of supply and demand. FEBS Lett. 476, 47–51.

Hofmeyr, J.H.S., Kacser, H., van der Merwe, K.J., 1986. Metabolic control analysis of moiety-conserved cycles. Eur. J. Biochem. 155, 631–641.

Irzyk, G.P., Fuerst, E.P., 1993. Purification and characterization of a glutathione S-transferase from Benoxacor-treated maize (*Zea mays*). Plant Physiol. 102, 803–810.

Jez, J.M., Cahoon, R.E., Chen, S., 2004. *Arabidopsis thaliana* glutamate-cysteine ligase. J. Biol. Chem. 279, 33463–33470.

Kacser, H., Acerenza, L., 1993. A universal method for achieving increases in metabolic production. Eur. J. Biochem. 216, 361–367.

Kim, S.J., Shin, Y.H., Park, E.H., Sa, J.H., Lim, C.J., 2003. Regulation of the gene encoding glutathione synthetase from fission yeast. J. Biochem. Mol. Biol. 36, 326–331.

Koebmann, B.J., Westerhoff, H.V., Snoep, J.L., Nilsson, D., Jensen, P.R., 2002. The glycolytic flux in *E. coli* is controlled by the demand for ATP. J. Bacteriol. 184, 3909–3916.

Korzeniewski, B., 1998. Regulation of ATP supply during muscle contraction: theoretical studies. Biochem. J. 330, 1189–1195.

Lee, S., Moon, J.S., Ko, T.S., Petros, D., Goldsbrough, P.B., Korban, S.S., 2003. Overexpression of *Arabidopsis* phytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol. 131, 656–663.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2000. Principles of Biochemistry. 3rd ed. Worth Publishers, Inc., New York 1152pp.

Löeffler, S., Hochberger, A., Grill, E., Winnacker, E.L., Zenk, M.H., 1989. Termination of the phytochelatin synthesis through sequestration of heavy metals by the reaction product. FEBS Lett. 258, 42–46.

Marrs, K.A., 1996. The functions and regulation of glutathione-S-transferases in plants. Annu. Rev. Plant. Physiol. Plant. Mol. Biol. 47, 127–158.

Meierjohann, S., Walter, R.D., Müller, S., 2002. Glutathione synthetase from *Plasmodium falciparum*. Biochem. J. 363, 833–838.

Meister, A., 1995. Glutathione metabolism. Methods Enzymol. 251, 3–13.

Mendes, P., 1993. GEPASI: A software package for modeling the dynamics, steady states and control of biochemical and other systems. Comput. Appl. Biosci. 9, 563–571.

Mendoza-Cózatl, D., Devars, S., Loza-Tavera, H., Moreno-Sánchez, R., 2002. Cadmium accumulation in the chloroplast of *Euglena gracilis*. Physiol. Plant. 115, 276–283.

Mendoza-Cózatl, D., Loza-Tavera, H., Hernández-Navarro, A., Moreno-Sánchez, R., 2005. Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, photosynthetic protists and plants. FEMS Microbiol. Rev., in press.

Meuwly, P., Rauser, W.E., 1992. Alteration thiol pools in roots and shoots in maize seedlings exposed to cadmium. Plant Physiol. 99, 8–15.

Moreno-Sánchez, R., Bravo, C., Westerhoff, H.V., 1999. Determining and understanding the control of flux. An illustration in submitochondrial particles of how to validate schemes of metabolic control. Eur. J. Biochem. 264, 427–433.

Nakazawa, R., Kato, H., Kameda, Y., Takenaga, 2002. Optimum assay of the activity of phytochelatin synthase from tobacco cells. Biol. Plant. 45, 311–313.

Noctor, G., Foyer, C., 1998. Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.

Noctor, G., Strohm, M., Jouanin, L., Kunert, K.J., Foyer, C.H., Rennenberg, H., 1996. Synthesis of glutathione in leaves of transgenic poplar (*Populus tremula* x *P. alba*) overexpressing γ-glutamylcysteine synthetase. Plant Physiol. 112, 1071–1078.

Noctor, G., Arisi, A.C., Jouanin, L., Valadier, M.H., Roux, Y., Foyer, C.H., 1997. The role of glycine in determining the rate of glutathione synthesis in poplar. Possible implications for glutathione production during stress. Physiol. Plant. 100, 255–263.

Noctor, G., Arisi, A.C., Jouanin, L., Foyer, C., 1998. Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol. 118, 471–482.

Olivier, B.G., Rohwer, J.M., Hofmeyr, J.H.S., 2005. Modelling cellular systems with PySCeS. Bioinformatics 21, 560–561.

Ortiz, D.F., Ruscitti, T., McCue, K.F., Ow, D.W., 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J. Biol. Chem. 270, 4721–4728.

Poolman, M.G., Fell, D.A., Thomas, S., 2000. Modelling photosynthesis and its control. J. Exp. Bot. 51, 319–328.

Pritchard, L., Kell, D.B., 2002. Schemes of flux control in a model of *Saccharomyces cerevisiae* glycolysis. Eur. J. Biochem. 269, 3894–3904.

Rea, P.A., Vatamaniuk, O.K., Rigden, D.J., 2004. Weeds, worms and more. Papain's long-lost cousin, phytochelatin synthase. Plant Physiol. 136, 2463–2474.

Riechers, D.E., Irzyk, G.P., Jones, S.S., Fuerst, P., 1997. Partial characterization of glutathione s-transferases from wheat (*Triticum* spp.) and purification of a safener-induced glutathione S-transferase from *Triticum tauschii*. Plant Physiol. 114, 1461–1470.

Rodríguez-Enríquez, S., Torres-Márquez, M.E., Moreno-Sánchez, R., 2000. Substrate oxidation and ATP supply in AS-30D hepatoma cells. Arch. Biochem. Biophys. 375, 21–30.

Rossignol, R., Letellier, T., Malgat, M., Rocher, C., Mazat, J.P., 2000. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. Biochem. J. 347, 45–53.

Salt, D.E., Rauser, W.E., 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol. 107, 1293–1301.

Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., Chet, I., Raskin, I., 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13, 468–474.

Segel, I.H., 1975. Enzyme Kinetics. Wiley, New York 957pp.

Teusink, B., Passarge, J., Reijenga, C.A., Esgalhado, E., van der Weijden, C.C., Schepper, M., Walsh, M.C., Bakker, B.M., van Dam, K., Westerhoff, H.V., Snoep, J.L., 2000. Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes? Testing biochemistry. Eur. J. Biochem. 267, 5313–5329.

Tsuji, N., Hirayanagi, N., Iwabe, O., Namba, T., Tagawa, M., Miyamoto, S., Miyasaka, H., Takagi, M., Hirata, K., Miyamoto, K., 2003. Regulation of phytochelatin synthesis by zinc and cadmium in marine green alga. Dunaliella Tertiolecta 62, 453–459.

Vallee, B.L., Ulmer, D.D., 1972. Biochemical effects of mercury, cadmium, and lead. Annu. Rev. Biochem. 41, 91–128.

van Dongen, J.T., Schurr, U., Pfister, M., Geigenberger, P., 2003. Phloem metabolism and function have to cope with low internal oxygen. Plant Physiol. 131, 1529–1543.

Vatamaniuk, O.K., Mari, S., Lu, Y., Rea, P.A., 2000. Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. J. Biol. Chem. 275, 31451–31459.

Vatamaniuk, O.K., Bucher, E.A., Ward, J.T., Rea, P.A., 2002. Worms take the "phyto" out of "phytochelatins". Trends Biotechnol. 20, 61–64.

Vatamaniuk, O.K., Mari, S., Lang, A., Chalasani, S., Demkiv, L.O., Rea, P., 2004. Phytochelatin synthase, a dipeptidiltransferase that undergoes multisite acylation with γ-glutamylcysteine during catalysis. J. Biol. Chem. 279, 22449–22460.

Youssefian, S., Nakamura, M., Orudgev, E., Kondo, N., 2001. Increased cysteine biosynthesis capacity of transgenic tobacco overexpressing an O-acetylserine (thiol) lyase modifies plant responses to oxidative stress. Plant Physiol. 126, 1001–1011.

Xiang, C., Oliver, D., 1998. Glutathione metabolic genes co-ordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10, 1539–1550.

Zhu, Y.L., Pilon-Smits, E.A.H., Tarun, A.S., Jouanin, L., Terry, N., 1999a. Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiol. 119, 73–79.

Zhu, Y.L., Pilon-Smits, E.A.H., Tarun, A.S., Weber, S.U., Jouanin, L., Terry, N., 1999b. Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol. 121, 1169–1177.

Otras publicaciones durante el Doctorado

Arch Microbiol (2005)
DOI 10.1007/s00203-005-0013-4

ORIGINAL PAPER

César Avilés · M. Eugenia Torres-Márquez
David Mendoza-Cózatl · Rafael Moreno-Sánchez

Time-course development of the cd^{2+} hyper-accumulating phenotype in *Euglena gracilis*

Received: 16 February 2005 / Revised: 5 May 2005 / Accepted: 17 May 2005

Abstract To determine the onset of the Cd^{2+}-hyperaccumulating phenotype in *Euglena gracilis*, induced by Hg^{2+} pretreatment (Avilés et al. in Arch Microbiol 180:1–10, 2003), the changes in cellular growth, Cd^{2+} uptake, and intracellular contents of sulfide, cysteine, γ-glutamylcysteine, glutathione and phytochelatins during the progress of the culture were analyzed. In cells exposed to 0.2 mM $CdCl_2$, the Cd^{2+}-hyperaccumulating phenotype was apparent only after 48 h of culture, as indicated by the significant increase in cell growth and higher internal contents of sulfide and thiol-compounds, along with a higher γ-glutamylcysteine synthetase activity. However, the stiochiometry of thiol-compounds/Cd^{2+} accumulated was similar for both control and Hg^{2+}-pretreated cells. Moreover, the value for this ratio was 2.1 or lower after 48-h culture, which does not suffice to fully inactivate Cd^{2+}. It is concluded that, although the glutathione and phytochelatin synthesis pathway is involved in the development of the Cd^{2+}-hyperaccumulating phenotype in *E. gracilis*, apparently other pathways and sub-cellular mechanisms are also involved. These may be an increase in other Cd^{2+} chelating molecules such as di- and tricarboxylic acids, phosphate and polyphosphates, as well as Cd^{2+} compartmentation into organelles.

Keywords *Euglena gracilis* · Cd^{2+} accumulation · Mercury · γ-glutamylcysteine · γ-glutamylcysteine synthetase · Glutathione · Phytochelatins.

C. Avilés · D. Mendoza-Cózatl · R. Moreno-Sánchez (✉)
Departamento de Bioquímica,
Instituto Nacional de Cardiología,
Juan Badiano No. 1, Col. Sección XVI,
Tlalpan, 14080 México

M. E. Torres-Márquez
Departamento de Bioquímica, Facultad de Medicina,
UNAM, Apdo Postal, 70-159,
04510 México D.F., México

Introduction

Cadmium is one of the most toxic heavy metals (Vallee and Ulmer 1972) and has become a serious contaminant of soils and water bodies, mainly due to anthropogenic activity and inappropriate methods of disposal (Clarkson 1995). Bioremediation, the use of organisms to remove toxic compounds including heavy metals from contaminated sites, is a fast-growing technology, which has several advantages over traditional methods (Salt et al. 1995; Dhankher et al. 2002). Currently, heavy metals are removed from water bodies by using ion exchange chromatography, which is expensive and inefficient for removing micromolar concentrations of heavy metals. As a consequence, development of alternative approaches for the treatment of heavy metal-contaminated ecosystems is required (Dhankher et al. 2002). Some varieties of plants have been used for removing Co^{2+}, Zn^{2+}, and Ni^{2+} from contaminated soils (Salt et al. 1995). Although such plants have the ability to accumulate extremely high concentrations of heavy metals, they tend to grow at slower rates, generating relatively little biomass and showing lower fitness in comparison to plants growing in non-heavy metal-polluted soils (Antonovics 1971; Dhankher et al. 2002). Thus, finding and developing plant and non-plant species that show resistance to heavy metals, which is accompanied by accumulation of the metal ion, may be helpful for designing bioremediation strategies. This approach is also being developed for Cd^{2+}-polluted water (Matsunaga et al. 1999).

Synthesis of phytochelatins (pcs), and enhanced synthesis of their metabolic precursors glutathione (GSH), γ-glutamylcysteine (γ-EC), cysteine (Cys) and sulfide, is a commonly used cellular response against Cd^{2+} stress in higher plants, some yeast and algae (see for a recent review Mendoza-Cózatl et al. 2005). The sulfate assimilation pathway generates sulfide and Cys. In turn, γ-EC is formed by γ-EC synthetase (γ-ECS) from glutamate. Cys and ATP and GSH is synthesized

Journal ID 203 Article ID 13 B
Dispatch: 21.6.05 Journal: 203 No. of pages: 10
Author's disk received ☑ Used ☑ Corrupted ☐ Mismatch ☐ Keyed ☐

Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta xx (2005) xxx – xxx

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

The bacterial-like lactate shuttle components from heterotrophic *Euglena gracilis*

Ricardo Jasso-Chávez[a,*], Israel García-Cano[a], Álvaro Marín-Hernández[a], David Mendoza-Cózatl[a], Juan Luis Rendón[b], Rafael Moreno-Sánchez[a]

[a]*Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano #1, Col. Sección XVI, Tlalpan, México, D. F. 14080, México*
[b]*Departamento de Bioquímica, Facultad de Medicina, UNAM, México*

Received 31 May 2005; received in revised form 22 July 2005; accepted 25 July 2005

Abstract

The structural and kinetic analyses of the components of the lactate shuttle from heterotrophic *Euglena gracilis* were carried out. Mitochondrial membrane-bound, NAD^+-independent D-lactate dehydrogenase (D-iLDH) was purified by solubilization with CHAPS and heat treatment. The active enzyme was a 62-kDa monomer containing non-covalently bound FAD as cofactor. D-iLDH was specific for D-lactate and it was able to reduce quinones of different redox potential values. Oxalate and L-lactate were mixed-type inhibitors of D-iLDH. Mitochondrial L-iLDH also catalyzed the reduction of quinones, but it was inactivated during the extraction with detergents. Both L-iLDH and D-iLDH were inhibited by the specific flavoprotein-inhibitor diphenyleneiodonium, suggesting that L-iLDH was also a flavoprotein. Affinity chromatography revealed that the *E. gracilis* cytosolic fraction contained two types of NAD^+-dependent LDH specific for the generation of D- and L-lactate (D-nLDH and L-nLDH, respectively). These two enzymes were tetramers of 126–132 kDa and showed an ordered bi–bi kinetic mechanism. Kinetic properties were different in both enzymes. Pyruvate reduction by D-nLDH was inhibited by its two products; the D-lactate oxidation was 40-fold lower than forward reaction. L-lactate oxidation by L-nLDH was not detected, whereas pyruvate reduction was activated by fructose-1, 6-bisphosphate, K^+ or NH_4^+. Interestingly, membrane-bound L- and D-lactate dehydrogenases with quinone reductase activity have been only detected in bacteria, whereas the activity of soluble D-nLDH has been identified in bacteria and some yeast. Also, FBP-activated L-nLDH has been found solely in lactic bacteria. Based on their similar kinetic and structural characteristics, a possible common origin among bacterial and *E. gracilis* lactic dehydrogenase enzymes is discussed.
© 2005 Published by Elsevier B.V.

Keywords: Membrane-bound lactate dehydrogenase; NAD^+-dependent lactate dehydrogenase; Mitochondrion; Energy metabolism

1. Introduction

The NAD^+-independent lactate dehydrogenases (iLDH) are enzymes widely distributed among bacteria and yeast [1,2]. Lactate oxidation is coupled to the respiratory chain and hence to the generation of a transmembrane proton gradient and ATP synthesis. In bacteria, the quinone pool is the physiological electron acceptor of iLDH, while in yeast, cytochrome *c* is the main acceptor. In bacteria, D-iLDH and L-iLDH, both stereo-specific enzymes, are localized in the inner cytoplasmic membrane, while in *Saccharomyces cerevisiae*, they are located in the mitochondrial intermembranal space [3,4]. In both kinds of microorganisms, the iLDHs are flavoproteins, but in yeast, D- and L-iLDH also have a cytochrome b_2. Many of these enzymes have been purified and their molecular and kinetic properties have been determined [1,2,5]. In bacteria and yeast, active D-iLDH is a 60–66 kDa monomer which contains one non-covalently bound FAD molecule [1,2]; in yeast, D-iLDH seems to be mainly associated with the methylglyoxal pathway [6]. On the other hand, L-iLDH is a 45-kDa monomer in bacteria and a 240-kDa homotetramer in yeast

* Corresponding author. Tel.: +52 555 573 2911; fax: +52 555 573 0926.
E-mail address: rjasso@incardio.com (R. Jasso-Chávez).

0005-2728/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.bbabio.2005.07.001

Flores Herrera O, Rendón Huerta E, Riveros Rosas H, Sosa Peinado A, Vázquez Contreras E, Velázquez López I (eds). **Mensaje Bioquímico, Vol XXIX**. Depto Bioquímica, Fac Medicina, Universidad Nacional Autónoma de México. Cd Universitaria, México, DF, MÉXICO. (2005). (http://bq.unam.mx/mensajebioquimico)
(*ISSN-0188-137X*)

EL ANÁLISIS DE CONTROL DE FLUJO COMO HERRAMIENTA EN LA MANIPULACIÓN DE VÍAS METABÓLICAS

Rafael Moreno Sánchez, Emma Saavedra, David Mendoza Cózatl y Sara Rodríguez Enríquez

Instituto Nacional de Cardiología
Departamento de Bioquímica
Juan Badiano No. 1, Colonia Sección XVI, Tlalpan, México, D.F. 14080.
rafael.moreno@cardiologia.org.mx; emma_saavedra2002@yahoo.com; dgmcozatl@yahoo.com.mx; rodsar@mail.cardiologia.org.mx

FLUX CONTROL ANALYSIS AS A TOOL FOR METABOLIC PATHWAY MANIPULATION

Abstract

Flux control analysis of a metabolic pathway allows the quantitative determination of the degree of control that a given enzyme (Ei) exerts on flux J (i.e., flux control coefficient C_{Ei}^{J}) and on the concentration of metabolites M (i.e., concentration control coefficient C_{Ei}^{M}), thus substituting the misleading, qualitative concept of rate limiting steps in metabolic pathways. In addition, it helps to understand (i) the underlying reasons by which a given enzyme exerts high or low control and (ii) why the pathway control does not reside in only one enzyme ("the rate limiting step"); instead, the control is shared by several enzymes and transporters.

Several different experimental approaches have been developed for the determination of C_{Ei}^{J}. For instance, titration of flux with specific inhibitors has been used in the control analysis of oxidative phosphorylation in isolated mitochondria and intact cells. Elasticity analysis is perhaps the most profusely approach used in many metabolic pathways, mainly glycolysis. This consists in the determination of the sensitivity of a given enzyme (or block of enzymes) towards the variation in the concentration of its substrates and products, under steady-state conditions of the pathway. If the enzyme activity does not significantly change with the variation in one of its ligands (low elasticity, ε_{Ei}^{J}), then the enzyme, transporter or block of enzymes is close to

METABOLISMO DEL GLUTATIÓN EN MICROORGANISMOS Y PLANTAS

David Mendoza Cózatl, César Avilés Rodríguez, Andrea Hernández Navarro[1], Herminia Loza Tavera[1] y Rafael Moreno Sánchez. Departamento de Bioquímica, Instituto Nacional de Cardiología. Juan Badiano 1, Tlalpan Sección XVI C. P. 14080, México D.F. [1]Facultad de Química, UNAM Cd. Universitaria, 04510, México D.F. Correo electrónico: cozatl@hotmail.com

Recibido: 13 de febrero de 2001. Aceptado: 19 de junio de 2001.

RESUMEN

El glutatión es el péptido de bajo peso molecular más abundante dentro de las células y se encuentra prácticamente en todos los organismos. La concentración de glutatión depende de diversos factores ambientales y su interconversión entre la forma reducida (GSH) y oxidada (GSSG) brinda un adecuado control del estado redox intracelular. Su biosíntesis es a través de la vía de asimilación del azufre, que a su vez está estrechamente relacionada con la síntesis de cisteína. El GSH funciona como el principal almacén intracelular de azufre y cisteína; participa además en el transporte de aminoácidos, metabolitos y en el procesamiento e inactivación de especies reactivas de oxígeno. En plantas y algas es esencial para la tolerancia a metales pesados. Poco se conoce sobre la regulación de su biosíntesis; sin embargo, la reciente clonación de los genes de las enzimas, que participan en la vía ha permitido un estudio mas detallado de su metabolismo. El determinar los mecanismos de regulación de la vía puede facilitar su manejo, lo cual podría tener diversas implicaciones en biotecnología.

PALABRAS CLAVE: Asimilación de azufre, cisteína, glutatión, fitoquelatinas, compartamentalización, metales pesados.

ABSTRACT

Glutathione is the most abundant low molecular weight thiol peptide inside the cells and occurs in all organisms. Glutathione concentration depends on diverse environmental factors, and its interconversion among the reduced (GSH) and oxidized form (GSSG) offers an appropriate control of the intracellular redox state. GSH biosynthesis involves the sulfur assimilation pathway, which is closely related with cysteine biosynthesis; in fact, GSH works as the main intracellular sulfur and cysteine storage. It also participates in the transport of amino acids, metabolites, and in the processing and inactivation of reactive oxygen species. In plants and algae, it is essential for heavy metals tolerance. Little is known about the regulation of GSH biosynthesis. However the recent cloning of the genes encoding the enzymes that participate in the pathway has allowed a more detailed study of its metabolism. The elucidation of the regulatory mechanisms of the pathway will allow its manipulation, which may have biotechnological application.

KEY WORDS: Sulfur assimilation, cysteine, glutathione, phytochelatins, compartmentation, heavy metals.

INTRODUCCIÓN

Los compuestos con azufre, como la cisteína, metionina, S-adenosilmetionina y glutatión son esenciales para el metabolismo de todas las células. La metionina, salvo pocas excepciones, es el aminoácido con el cual se inicia la biosíntesis de proteínas. La cisteína tiene una función crucial en la estructura, estabilidad y función catalítica de enzimas. La S-adenosilmetionina interviene en la transferencia de grupos metilo y biosíntesis de poliaminas. El glutatión (γ-Glu-Cys-Gly; GSH) tiene diversas funciones dentro del metabolismo. Su capacidad de mantener el estado redox intracelular, además de su participación en el transporte de aminoácidos y destoxificación de xenobióticos, hacen de ésta una molécula esencial. El GSH mantiene enzimas y otros componentes celulares en estado reducido y es, además, el principal almacén y transportador de sulfuro y cisteína [1]. Dentro de la célula, más del 90% del azufre no-proteíco está en forma de GSH y la mayoría se encuentra en forma reducida [2]. El GSH es el producto final de una serie de reacciones que constituyen la vía metabólica de asimilación de azufre y síntesis de cisteína.