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CARACTERIZACIÓN BIOQUÍMICA Y PAPEL FISIOLÓGICO DE LA OXIDASA ALTERNA EN *Euglena gracilis*

T E S I S

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RESUMEN

Euglena gracilis posee una cadena respiratoria que esta integrada por componentes clásicos tales como la ubiquinona-9, el citocromo *c* y los complejos enzimáticos I, II, III y IV que son característicos de los mamíferos y otros organismos. Sin embargo, también posee otros complejos alternativos como dos lactato deshidrogenasas isómero-específicas, una rodoquinona-9, un complejo tipo-bc_1 alternativo al complejo III y una oxidasa terminal alternativa (AOX) al complejo IV.

Durante mi trabajo de Maestría detectamos la presencia de la AOX en *E. gracilis* mediante réplica Western y, con base en la sensibilidad a los inhibidores reportados para esta enzima, sugerimos que posee semejanzas importantes con la AOX descrita para otros eucariontes.

El trabajo de esta tesis se ha enfocado en analizar las características bioquímicas y la relevancia funcional de la oxidasa alterna en este protista.

Uno de los aspectos más estudiados en este campo es la función que la AOX desempeña en cada organismo. Su presencia en las plantas termogénicas tiene claramente un fin de producción de energía calórica, mientras que en los hongos patógenos, la actividad de la AOX le permite sobrevivir al ataque del hospedero mediado por el óxido nítrico, el cual inhibe al complejo IV. Sin embargo, en *E. gracilis* nosotros hipotetizamos que su función está relacionada con la respuesta al estrés oxidativo generado en algunas condiciones adversas a las cuales se suele enfrentar en su ambiente natural.

Inicialmente, los cultivos a los cuales se les adicionó una concentración tóxica de inhibidores respiratorios como el cianuro o la antimicina, mostraron un daño generalizado e impidió una evaluación confiable de la AOX. Por esta razón decidimos utilizar condiciones que pueden existir en el hábitat de *E. gracilis*.

La primera condición utilizada fue la exposición a temperatura baja. Los resultados mostraron que el frío afecta la cadena respiratoria a nivel del complejo III, sin que otro componente respiratorio se viera disminuído. En contraste, encontramos un aumento importante en la actividad y en la concentración de la AOX, y también en la actividad de la succinato deshidrogenasa (SDH), en el contenido del quinol y en el tamaño de la poza de las quinonas presentes en *E. gracilis* (ubiquinona-9 y rodoquinona-9).

El rodoquinol es donador de electrones en la reducción anaeróbica de fumarato en otros organismos. Sin embargo, la concentración tan alta de este compuesto, así como sus variaciones durante la exposición al frío, generó la interrogante del papel que tiene la rodoquinona en la transferencia de electrones aeróbica en *E. gracilis*. Los resultados mostraron una transferencia de electrones al fumarato en condiciones anaeróbicas y aeróbicas. Más aún, se encontró que la poza de rodoquinona se reduce específicamente con los electrones provenientes de la oxidación del D-lactato a través de la enzima isómero-específica D-lactato deshidrogenasa (D-iLDH) en las mitocondrias de este protista.

Por otro lado, mediante el análisis de diversas actividades mitocondriales usando a la rodoquinona endógena purificada como sustrato (en su estado reducido), se logró obtener la reducción del citocromo *c* a través de la actividad de los complejos III (sensible

a antimicina) y tipo-III (sensible a mixotizazol). El análisis cinético mostró que la afinidad por el rodoquinol-9 era menor que por el ubiquinol-9.

En conclusión, la rodoquinona es reducida por la D-iLDH y oxidada principalmente por la fumarato reductasa y también transfiere electrones a la cadena respiratoria aeróbica pero con menor eficiencia catalítica.

También se utilizó otra condición de estrés para evaluar a la AOX: la exposición a cadmio, uno de los metales pesados más tóxicos. Se sabe que *E. gracilis* tolera concentraciones tóxicas de metales pesados como mercurio, cromo, cadmio, plomo y zinc. Aunque se han evaluado diferentes aspectos relacionados con la entrada y acumulación del cadmio, así como la respuesta de defensa mediada por grupos tioles, poco se sabe de las respuestas antioxidantes y de la resistencia que se activan durante este evento. La hipótesis planteada sobre la función de la AOX para limitar el estrés oxidativo estableció que en condiciones de exposición a cadmio donde se genera estrés oxidativo, esta enzima limita la producción de las especies reactivas del oxígeno generado en la mitocondria.

Al exponer a las células de *E. gracilis* a concentraciones tóxicas de cadmio se observó un aumento en la lipoperoxidación, un aumento en la oxidación de la poza del glutatión, un aumento en los metales que son promotores de estrés oxidativo (Fe y Cu) y un aumento de las enzimas antioxidantes.

En contraste, encontramos que, aunque la respiración celular no varió, ésta fue más resistente al cianuro. Debido a que el efecto inhibitorio del cadmio ocurrió principalmente a nivel de los complejos III y IV la respiración mitocondrial fue soportada principalmente por la actividad de la AOX. La concentración de la AOX también se

incrementó. Encontramos también un aumento en la poza de la ubiquinona y un aumento en la actividad de la SDH similar a lo encontrado en la condición de exposición a frío.

Concluimos que en *E. gracilis*, el aumento en la cantidad y actividad de la AOX constituye parte de la respuesta celular a la exposición a condiciones estresantes, a través de una modulación en la acumulación de electrones y de una disminución en la reducción de los componentes respiratorios clásicos y por lo tanto, de la generación de especies reactivas de oxígeno.

ABSTRACT

Euglena gracilis has a respiratory chain composed by typical components. However, several alternative components have been described: isomer-specific lactate dehydrogenases, rhodoquinone-9, a bc_1-like complex and an alternative oxidase

Previously, the reaction in *E. gracilis* by using an antibody against AOX and the sensitivity to compounds reported as AOX inhibitors suggested that the *E. gracilis* enzyme shares some features with AOX from other eukaryotes.

In this work, we focus in the analysis on the biochemistry and functional relevance of the AOX in *E. gracilis*.

The function of the AOX has been one of the most studied topics. It is present in thermogenic plants, in pathogenic fungus, etc. In addition, a relevant function in the response to the oxidative stress during some adverse conditions is proposed in *E. gracilis*.

To test this hypothesis, we decided to use some stressful conditions to which *E. gracilis* can be physiologically exposed.

First, a model of low temperature was used. The results showed a diminution of the complex III activity. AOX increased its concentration and activity as well as SDH, quinol content and quinone size pool (rhodoquinone-9 and ubiquinone-9).

Rhodoquinol donate electrons for anaerobic reduction of fumarate. But the high content of this compound in *Euglena* as well its variations during stress conditions arose the question about the role of this quinone in the mitochondria of this protist. The results showed that fumarate can be reduced during aerobic or anaerobic conditions. Moreover, we found a specific reduction of rhodoquinone by D-lactate oxidation.

On the other hand, the use of purified rhodoquinone showed an electron transfer for cytochrome c reduction by complex III and bc_1-like complex. The affinity of these components for rhodoquinone was lower than for ubiquinone.

The third condition was the exposition to cadmium. It is well known that *E. gracilis* tolerates toxic concentrations of mercury, chromium, cadmium, lead and zinc. Several studies with cadmium have shown the mechanisms involved in the tolerance and resistance to this metal. However, little is known about the antioxidant responses generated from the toxicity of cadmium and also the role of the AOX in this condition.

The *E. gracilis* exposure to cadmium increased the level of lipoperoxidation, the glutathion pool and ROS-promoters: Fe and Cu. In addition, we found and increase in the antioxidant enzymes activities.

In this condition, the cellular respiration was not modified but it was more resistant to cyanide inhibition. Mitochondrial respiration diminished slightly but mainly supported by AOX. The ubiquinone pool and SDH activity increased as it was found in the model of cold exposure.

With these results we conclude that AOX has an important role in the cellular response when the cells are exposed to stressful conditions by modulating the electron flux and by limiting the reduction level of different components thus avoiding ROS production.

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ABREVIATURAS

ATP: trifosfato de adenosina

AOX : oxidasa alterna

DHAP : di-hidroxiacetona fosfato

G3P : glicerol 3-fosfato

G3P DH : glicerol 3-fosfato deshidrogenasa

CI : complejo I, NADH-quinona oxidorreductasa

bc_1 : complejo III, quinol-citocromo c oxidorreductasa

NADH: nicotinamida-adenín dinucleótido

NaR : nitrato reductasa

NiR : nitrito reductasa

UQ : ubiquinona

RQ : rodoquinona

SO: sulfuro oxidasa

Xe: xenobiótico

DH: deshidrogenasa

AMP: monofosfato de adenosina

GMP: monofosfato de guanosina

ERO: especies reactivas de oxígeno

n-PG: n-propil galato

SHAM: ácido salicil hidroxámico

ON: óxido nítrico

CAPÍTULO I

INTRODUCCIÓN

1.- GENERALIDADES SOBRE LAS CADENAS RESPIRATORIAS

- LA CADENA RESPIRATORIA CLÁSICA

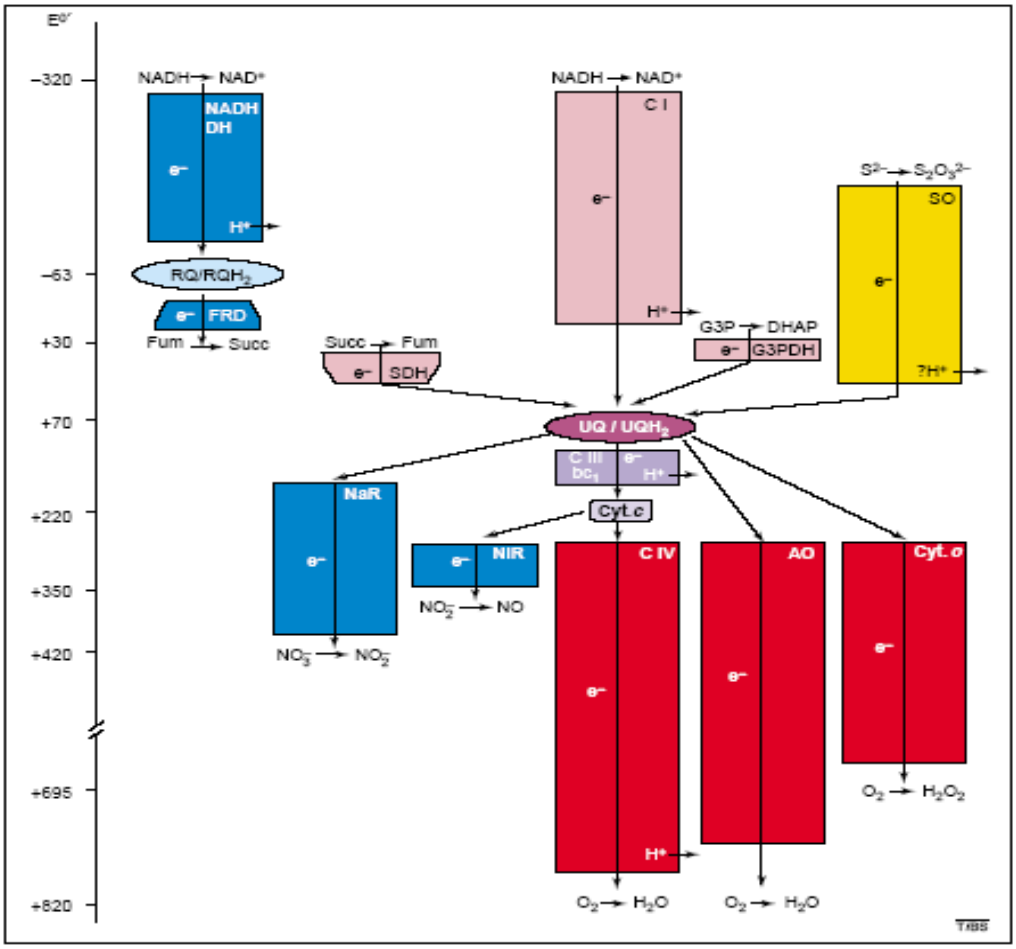
Los organismos obtienen la energía necesaria para el funcionamiento de su metabolismo a través de la síntesis de ATP. Uno de los procesos más eficientes para lograrlo es la transferencia electrónica a través de las reacciones de óxido-reducción entre proteínas y moléculas orgánicas. Esta serie de reacciones se conoce como cadena transportadora de electrones.

Una de las cadenas más estudiadas es la de las mitocondrias de los mamíferos [1], la cual puede ser considerada como una vía sencilla y lineal a partir de la poza de quinonas y que está constituída por citocromos. Para efecto de este trabajo, tal vía se denominará vía clásica.

- LAS CADENAS RESPIRATORIAS COMPLEJAS

La caracterización de la vía clásica, como la presente en células de mamíferos, ha mostrado una vía metabólica relativamente simple. Sin embargo, en el resto de los organismos como las plantas, los hongos (levaduras y hongos filamentosos), los protistas, así como las bacterias existen cadenas respiratorias más complejas, donde existen ramificaciones que proporcionan la flexibilidad necesaria para sobrevivir en condiciones

ambientales extremas [2, 3, 4]. En la Fig. 1 se ejemplifican algunos de los componentes reportados en diferentes modelos de estudio.



Tomado de AGM Tielens *et al*. TIBS (2002)

Figura 1.- Ejemplos de diferentes componentes alternativos presentes en cadenas respiratorias mitocondriales.

Una de las características esenciales de estas ramificaciones alternas es la resistencia a compuestos que usualmente inhiben a componentes clásicos de la vía. Esto ha permitido que el uso de estos inhibidores sirva para identificar y caracterizar

parcialmente los componentes alternativos. Por ejemplo, la oxidación del NADH resistente a rotenona en plantas sugirió la presencia de una NADH deshidrogenasa alterna, insensible a este inhibidor.

La diversidad de estos componentes alternativos es amplia. Se han encontrado varios grupos de enzimas en diferentes sitios de la cadena respiratoria: en la oxidación de sustratos (por ejemplo las deshidrogenasas para el L- lactato, D-lactato) [5], en la oxidación de quinol (by-passes) [6] o como oxidasas terminales alternas [4, 7, 8]. Además, en cada grupo puede existir una variación genética, estructural y mecanística. Por ejemplo, las oxidasas terminales alternas de las bacterias pueden tener diferentes grupos hemo [4], mientras que en los eucariontes las oxidasas terminales alternas carecen de este grupo prostético.

2.- *Euglena gracilis* Y SU CADENA RESPIRATORIA

Se ha utilizado *Euglena gracilis* como un organismo modelo del género *Euglena* y de otros organismos eucariontes debido a su unicelularidad y al rendimiento que puede obtenerse de los organelos como mitocondrias y cloroplastos. Su facilidad de cultivo también ha contribuido al uso de este organismo.

Euglena gracilis es un protista fotosintético de vida libre que habita en una gran variedad de condiciones ambientales. Se ha encontrado en agua dulce, en ambientes con un pH ácido o más alcalino, y en ambientes que tienen variaciones importantes en la concentración del oxígeno. También se han encontrado otras especies de *Euglena* en el agua salada, en suelos y en la nieve [9]. Ademas, la investigación realizada por algunos

grupos de investigación, incluyendo el nuestro, ha mostrado la franca resistencia de *Euglena gracilis* a altas concentraciones de metales pesados como mercurio y cadmio [10].

En general, podemos decir que *Euglena* posee características que le permiten habitar en diferentes ambientes, algunos son tan extremos que pueden generar estrés oxidativo (por ejemplo, la exposición a una baja temperatura o a los metales pesados).

Dentro de las características que pueden estar involucradas en la tolerancia a los metales, se ha evaluado el mecanismo de captación y acumulación del cadmio en el cloroplasto a través de compuestos con grupos tioles como el glutatión y las fitoquelatinas [11]. También, se ha sugerido que existe un mecanismo similar en la mitocondria [12].

Alternativamente a estos mecanismos existe otra parte que es de nuestro interés y que puede completar el panorama respecto a la tolerancia a las condiciones extremas y por lo tanto ayudar a entender la gran flexibilidad de este organismo.

Por la información recopilada por Buetow [13] se sabe desde hace tiempo que *Euglena gracilis* tiene diferencias importantes en sus mitocondrias con respecto a las de mamíferos. Con base en diversos análisis bioquímicos [14], se ha modelado una cadena respiratoria mitocondrial compleja (Fig. 2). Como puede observarse, se encuentra conformada por enzimas respiratorias tipo mamífero y también por diferentes componentes alternos. Los complejos correspondientes a la vía clásica poseen características similares a los presentes en cadenas respiratorias de mamíferos, con la excepción del complejo III, el cual posee sensibilidad a la antimicina y a la estigmatelina (como se espera de un complejo III clásico), pero atípicamente es resistente al mixotiazol, otro de los inhibidores clásicos de este complejo.

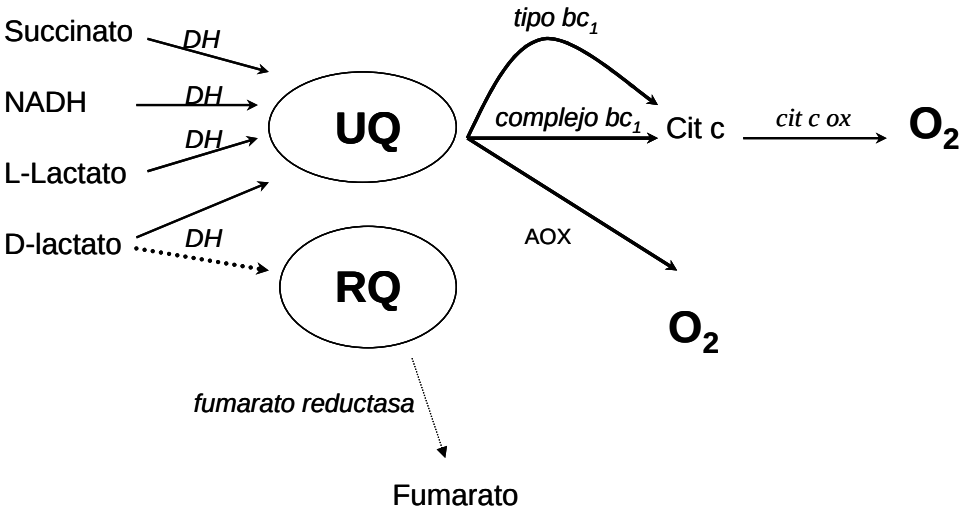


Figura 2.- Cadena respiratoria de *Euglena gracilis*.

-LOS COMPONENTES ALTERNOS DE LA CADENA RESPIRATORIA DE *Euglena gracilis*

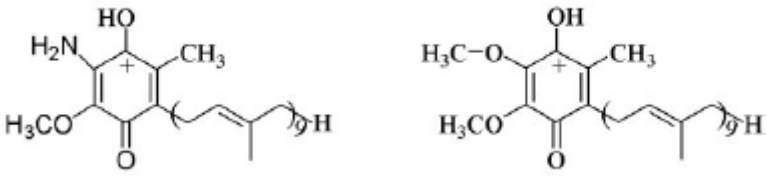
Dentro de los componentes alternos podemos citar cinco muy importantes: L- y D-lactato deshidrogenasas mitocondriales independientes de piridin nucleótidos, la rodoquinona, el componente alterno tipo-complejo III y la oxidasa alterna.

L- y D-lactato deshidrogenasa: la mitocondria de *E. gracilis* es capaz de oxidar L- y D-lactato. Este tipo de actividades sólo se han detectado y analizado en este organismo [15], en levaduras [16] y en bacterias [5]. Trabajando con la cepa heterotrófica (no fotosintética) de *E. gracilis*, Jasso- Chávez y cols. [15] lograron identificar que estos sustratos son oxidados en la mitocondria por enzimas isómero-específicas.

La D-iLDH tiene la particularidad de resistir altas temperaturas (hasta los 70°C), lo que permitió la purificación de esta enzima con respecto a otras proteínas. Se determinó que ambas enzimas eran capaces de reducir diferentes quinonas comerciales, análogas de la ubiquinona (de alto potencial redox), además de la tetraclorohidroquinona, una quinona sintética de bajo potencial redox [17].

Rodoquinona: Adicionalmente a la ubiquinona-9 (quinona de alto potencial), *E. gracilis* posee rodoquinona-9 [18, 19] una quinona de bajo potencial (Fig. 3), presente de forma característica en rodobacterias y nemátodos [20, 21, 22]. Esta quinona en su estado reducido sirve de donador de electrones para la reducción anaeróbica de fumarato para formar succinato [20]. Si bien se ha considerado a *E. gracilis* como un organismo facultativo anaerobio, debido a que tolera bajas concentraciones de oxígeno, es claro también que este compuesto se encuentra presente en concentraciones importantes en células cultivadas o incubadas en condiciones aeróbicas [19], lo que despierta la interrogante sobre la verdadera participación de este compuesto en la cadena respiratoria de este protista.

(A) (B)



E_0' -63 mV E_0' +70 mV

Figura 3.- Quinonas presentes en *E. gracilis*. A: Rodoquinona-9, B: Ubiquinona-9

Componente alterno tipo complejo III (by-pass): Atípicamente, el complejo III de *E. gracilis* posee resistencia a mixotiazol, uno de los inhibidores clásicos del complejo III de mamíferos [23]. Las preparaciones purificadas de este complejo mostraron actividades, medidas como reducción de citocromo *c*, totalmente sensibles a antimicina y a estigmatelina. Sin embargo, en mitocondrias frescas se detectó que la reducción de citocromo c se inhibía por antimicina un 60 % aproximadamente, mientras que el resto de la actividad era abatido con mixotiazol. Ambos componentes son inhibidos por estigmatelina, un inhibidor más del complejo III. También es importante mencionar que el quinol alimenta directamente a este complejo alterno y es capaz de reducir al citocromo c y no a otro componente, de manera que el paso sugiere un salto o vía paralela a nivel del complejo III como se muestra en la Fig. 2. Un análisis espectrofotométrico previo de citocromos en esta misma cepa [19] no reveló la presencia de algún componente citocrómico diferente a los citocromos *b*, *c*, c_1, o aa_3 correspondientes a los complejos III y IV clásicos. Estos resultados sugirieron que el componente alterno podría pertenecer a una población adicional de complejo III, pero con diferente sensibilidad a inhibidores.

-LA OXIDASA ALTERNA DE *E. gracilis*

Por último, otro de los componentes presentes en la cadena respiratoria es una oxidasa terminal alterna (AOX). Inicialmente se identificó como una respiración resistente a cianuro. Además, durante mis estudios de la Maestría, se evidenció en *E. gracilis* heterotrófica la sensibilidad de esta actividad a otro tipo de compuestos como son los alquil galatos, ácidos hidroxámicos, disulfiram, etc. Este tipo de enzimas respiratorias

terminales sensibles a estos compuestos se encuentra presente en mitocondrias de otros organismos eucariontes, como en plantas y hongos [24, 25, 26].

En *E. gracilis*, cultivada en condiciones no estresantes, la AOX es responsable del 10-15 % de la respiración total que denominaremos como “capacidad de la oxidasa alterna”, debido a que la medición en presencia de cianuro solo refleja la capacidad máxima de transferencia de electrones y no su actividad fisiologica [27].

Utilizando un anticuerpo dirigido contra la AOX del alga *Chlamydomonas reinhardtii,* pudimos identificar previamente una banda de 38 kDa tanto en la cepa heterotrófica como en la fotosintética, que corresponde al rango de peso molecular descrito para esta enzima [28].

-CARACTERÍSTICAS BIOQUÍMICAS DE OTRAS AOX

Desde 1929 las AOX se han identificado por la actividad respiratoria resistente al cianuro, potente inhibidor del complejo IV [24]. Se sabe también que éste no participa en la fosforilación oxidativa al no translocar protones [29]. Como se mencionó anteriormente, la información más completa y detallada se encuentra en los modelos de plantas, de donde ha sido purificada y caracterizada bioquímica y molecularmente [24, 25]. También existe información sobre esta enzima en levaduras, hongos filamentosos y sobretodo en tripanosomátidos [30], en donde se ha propuesto como un blanco terapéutico al no estar presente en el hospedero.

Bioquímicamente, se sabe que la AOX es una enzima que carece de grupo hemo, diferenciándola de las oxidasas terminales alternas reportadas para bacterias [24].

También posee un centro de reacción bi-férrico semejante al presente en las ácido graso desaturasas [31, 32]. Esto ha sugerido un origen evolutivo común a partir de enzimas como ferritinas, mono-oxigenasas y desaturasas [32].

La AOX es una enzima adosada en la membrana interna mitocondrial de un peso molecular que se ha descrito estar entre 34 y 38 kDa, según diferentes reportes [28, 33]. En plantas se sabe que la forma activa es una enzima dimérica cuyo residuo de cisteína 78 regula su actividad [34]. Otro mecanismo involucra la activación por α-cetoácidos, principalmente piruvato, mediante la formación de un tiohemiacetal cuando el residuo de cisteína está reducido [35].

A diferencia de la enzima en plantas, el resto de los organismos posee una forma activa monomérica donde se ha observado que la adición de AMP o GMP estimula la actividad [36, 37], aunque el mecanismo por el cual estos nucleótidos activan se desconoce. También se ha determinado que el pH óptimo de la AOX es aproximadamente 6.8, más ácido que el del complejo IV, que es 7.3 [38]. En cuanto a la temperatura, existen algunos estudios en plantas [39-42], en donde la baja temperatura revela un aumento en la actividad de la AOX, lo que ha llevado, junto con mediciones de enzimas antioxidantes, a algunos autores a proponer que una de sus funciones importantes es la del manejo del estrés oxidativo [24, 43]. Algunas de las características más relevantes de la AOX se muestran en la Tabla 1.

Tabla 1 Características generales de las AOX

Modelo	Especie activa	Sensibilidad a n-PG y SHAM	Regulación	Función
Planta [1]	Dímero	+	Estado redox	Termogénesis, estrés oxidativo
Hongos [2]	Monómero	+	AMP	Respuesta al estrés oxidativo, favorece la generación de ATP, mecanismo de defensa contra NO
Trypanosomátidos[3]	Monómero	+	GMP	Estadio de vida, estrés oxidativo
E. gracilis	¿?	+	¿?	¿?

Ref. [1] 25, 31, 34; [2] 7, 46; [3] 30.

-FUNCIÓN DE LAS AOX

Esta enzima fue inicialmente descrita en las plantas termogénicas de la familia *Araceae* para el proceso de polinización, en las cuales la actividad de la AOX otorga un aumento significativo en la temperatura de la planta y en la posterior volatilización de compuestos que atraen a insectos polinizadores [44]. Sin embargo, la presencia de esta enzima en plantas y otros organismos no termogénicos sugirió una función(es) adicional(es) a las ya reportadas. Se determinó que en el epimastigote de *Trypanosoma* esta enzima cataliza la reducción de oxígeno cuando su metabolismo depende de la

glucólisis [30], mientras que para el promastigote se sugiere que esta enzima es relevante para limitar el estrés oxidativo [45].

Actualmente, la presencia de la AOX en modelos en los que se genera estrés oxidativo como en plantas expuestas a baja temperatura, en hongos patógenos expuestos a la respuesta oxidante del tejido infectado [46, 47], o cuando la vía clásica se encuentra inhibida o limitada llevando a la acumulación de electrones sobre todo en la poza de quinonas, ha sugerido que la actividad de la AOX es relevante para limitar la generación de especies reactivas del oxígeno (EROs) mediante el drenaje de los electrones acumulados.

En *E. gracilis* resulta interesante analizar si ésta es la función que desempeña la AOX en sus mitocondrias debido a que, sorpresivamente, este protista carece de algunas de las respuestas antioxidantes más importantes de las células como la catalasa (CAT) [48] y la superóxido dismutasa (SOD) mitocondrial [49]. Se sabe que posee un sistema antioxidante basado principalmente en el ciclo de reducción de ascorbato y glutatión. Además, posee altas concentraciones de glutatión, α-tocoferol y carotenoides. También contiene glutatión S-transferasas, así como un mecanismo de resistencia a cadmio mediado por fitoquelatinas (derivados de glutatión) [11].

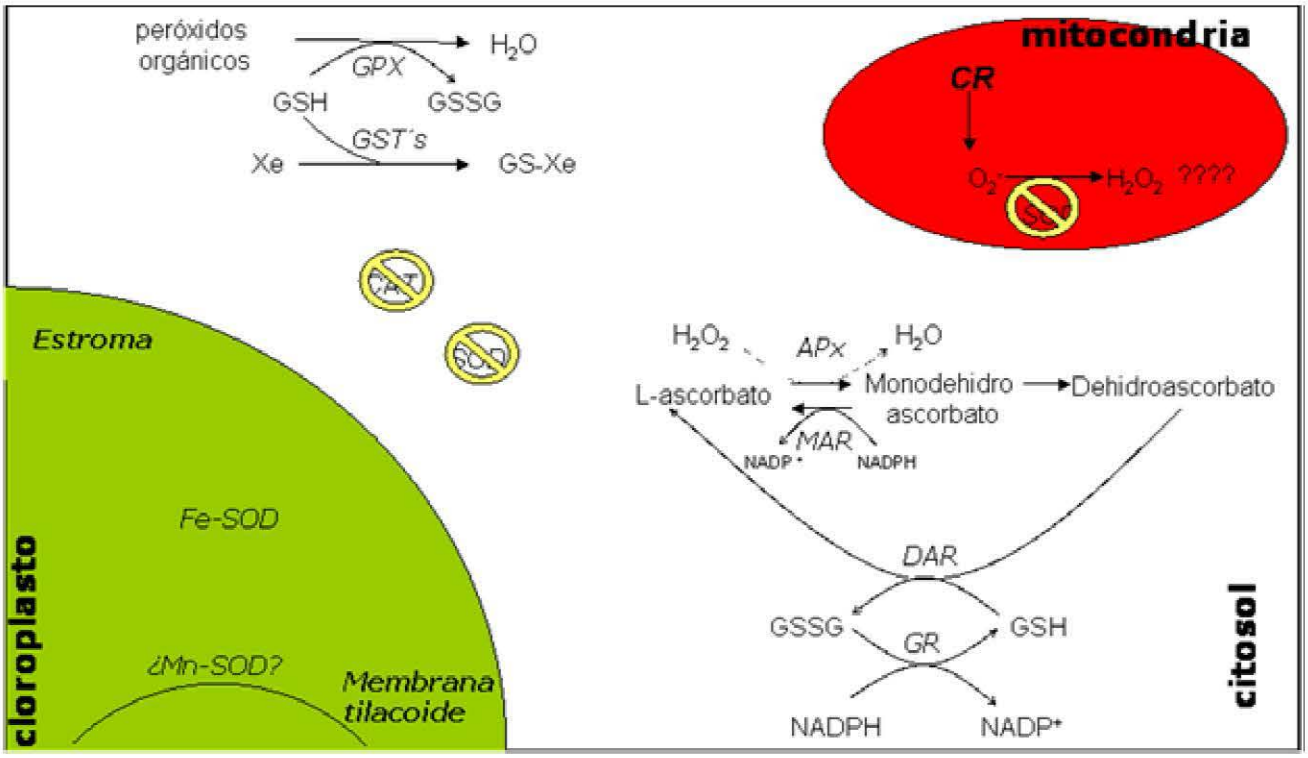


Figura 4.- Sistemas antioxidantes reportados en *E. gracilis* (Modelo realizado con base en las referencias 48 y 49).

De esta manera, es posible que en este protista la ausencia de tales enzimas pueda ser compensada por otros mecanismos (ascorbato peroxidasa, APx; glutatión peroxidasa, GPx; glutatión; ascorbato), así como por vías que limiten la producción de EROs (vía respiratoria ramificada).

-ESTRÉS OXIDATIVO CON FRÍO Y CON CADMIO

Existen varios estudios que muestran el aumento de estrés oxidativo en condiciones de exposición a temperaturas menores a 15 °C. El grado de estrés se evalúa como un

aumento en los niveles de hidroperóxidos o lipoperóxidos [50, 51], en la concentración de compuestos antioxidantes como ubiquinona y α-tocoferol y en la actividad de las enzimas antioxidantes APx, SOD, CAT y glutatión reductasa (GR) [50-54]. Respecto a la AOX, se ha documentado el aumento en la actividad de esta enzima por efecto del frío en diferentes modelos de plantas [52, 55] y en el de la amiba *A. castellanii* [56].

La determinación de EROs en algunos organismos incluyendo *E. gracilis* [58-60] y la estimulación de respuestas antioxidantes (CAT, SOD y enzimas pertenecientes al ciclo de ascorbato-glutatión como la GR) [57, 61] han mostrado que el cadmio genera estrés oxidativo. Algunos de los principales estudios son aquellos donde se analiza la respuesta celular a la exposición a este metal, en los cuales la activación en la síntesis de fitoquelatinas (polímeros de glutatión) promueve el secuestro del metal, impidiendo el daño a la célula [11, 57].

Sin embargo, en ninguno de ellos existe información donde se relacione a éste u otro metal con la AOX.

REFERENCIAS

1. – DG Nicholls y SJ Ferguson, Respiratory chains, en: Bioenergetics 3, Capítulo 5 por DG Nicholls y SJ Ferguson, Academic Press, 2002, Elsevier, UK, pág. 89.
2.- JW de Gier, Lübben M, Reijnders WNM, Tipker CA, DJ Slotboom, RJM van Spanning, AH Stouthamer and J van der Oost (1994) The terminal oxidases of *Paracoccus denitrificans*, Mol Microbiol 13: 183 -196.
3.- EJ Helmerhorst, MP Murphy, RF Troxler and FG Oppenheim (2002) Characterization of the mitochondrial respiratory pathways in *Candida albicans*, Biochim Biophys Acta 1556: 73 - 80.
4. - RK Poole and GM Cook (2000) Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation, Adv Microbiol Physiol 43: 165- 224.
5. - EI Garvin (1980) Bacterial lactate dehydrogenases, Microbiol Rev 44: 106- 139.
6. - G Brasseur, P Bruscella, V Bonnefoy and D Lemesle.Meunier (2002) The bc_1 complex of the iron-grown acidophilic chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* functions in the reverse but not in the forward direction. Is there a second bc_1 complex? Biochim Biophys Acta 1555: 37- 43.
7. - T Joseph-Horne, DW Hollomond and PM Wood (2001) Fungal respiration: a fusion of standard and alternative components, Biochim Biophys Acta, 1504: 179- 195.
8. - A McDonald and G Vanlerberghe (2004) Branched mitochondrial electron transport in the Animalia: presence of alternative oxidase in several animal phyla, IUBMB Life 56: 333- 341.
9. - JB Lackey, Ecology of *Euglena*, en: The biology of *Euglena*, editado por DE Buetow, Volumen I (1969), Academic Press, USA, pág. 28- 45.
10. - S Devars, R Hernández and R Moreno-Sánchez (1998) Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by exposure to mercury or cadmium, Arch Environ Contam Toxicol 34: 128- 135.
11. - DG Mendoza-Cózatl and R Moreno-Sánchez (2005) Cd^{2+} transport and storage in the chloroplast of *Euglena gracilis*, Biochim Biophys Acta 1706: 88-97.

12.- C Avilés, H Loza-Tavera, N Terry and R Moreno-Sánchez (2003) Mercury pretreatment selects an enhanced cadmium-accumulating phenotype in *Euglena gracilis*, Arch Microbiol 180: 1-10.

13.- WF Danforth, Respiration, en: The biology of *Euglena*, editado por DE Buetow, Volumen II (1968) Academic Press, USA, pág. 55- 72.

14.- R Moreno-Sánchez, R Covián, R Jasso-Chávez, S Rodríguez-Enríquez, F Pacheco-Moisés and ME Torres-Márquez (2000) Oxidative phosporylation supported by an alternative respiratory pathway in mitochondria from *Euglena*, Biochim Biophys Acta 1457: 200-210.

15.- R Jasso-Chávez, ME Torres- Márquez and R Moreno- Sánchez (2001) The membrane- bound L- and D- lactate dehydrogenase activities in mitochondria from *Euglena gracilis*, Arch Biochem Biophys 390: 295- 303.

16. - CA Fewson, DP Baker, RM Chalmers, JN Keen, ID Hamilton, AJ Scout and M Yasin (1993) Relationship amongst some bacterial and yeast lactate and mandelate dehydrogenases, J Gen Microbiol 139: 1345- 1352.

17. - R Jasso-Chávez, I García-Cano, A Marín-Hernández, D Mendoza-Cozatl, JL Rendón and R Moreno-Sánchez (2005) The bacterial-like lactate shuttle components from heterotrophic *Euglena gracilis*, Biochim Biophys Acta 1709: 181-190.

18. - R Powls and FW Hemming (1966) The properties and significance of rhodoquinone-9 in autotrophic and etiolated cultures of *Euglena gracilis* var. bacillaris, Phytochemistry 5: 1235-1247.

19. - Norma A Castro Guerrero (2002) Tesis de Maestría, Caracterización de los componentes resistentes a cianuro y antimicina de la cadena respiratoria de *Euglena gracilis*, Facultad de Química, UNAM.

20. - C Hägerhäll (1997) Succinate: quinone oxidoreductases, variations on a conserved theme, Biochem Biophys Acta 107-141.

21. - S Takamiya, K Kita, H Wang, PP Weinstein, A Hiraishi, H Oya and T Aoki (1993) Developmental changes in the respiratory chain of *Ascaris* mitochondria, Biochim Biophys Acta 1141: 65- 74.

22. - H Miyadera, A Hiraishi, H Miyoshu, K. Sakamoto, R. Mineki, K. Murayama, K.V. Nagashima, K. Mtsuura, S. Kojima, K. Kita (1997) Complex II from phototrophic purple bacterium *Rhodoferax fermentans* displays rhodoquinol-fumarate reductase, Parasitology 115 177-182.
23. - Covián García Raúl Miguel (1999) Tesis de Licenciatura, Purificación y caracterizacion del complejo bc_1 de mitocondrias de *Euglena gracilis*, UNAM.
24. - AM Wagner and AL Moore (1997) Structure and function of the plant alternative oxidase: Its putative role in the oxygen defense mechanism, Biosc Rep 17: 319- 333.
25. - AM Wagner and K Krab (1995) The alternative respiration pathway in plants: role and regulation, Physiol Plant 95: 318- 325.
26. - M Guerin and NM Camougrand (1994) Partitioning of electron flux between the respiratory chains of the yeast *Candida parapsilosis*: parallel working of the the two chains, Biochim Biophys Acta 1184: 111- 117.
27. - AE McDonald, SM Sieger and GC Vanlerberghe (2002) Methods and approaches to study plant mitochondrial alternative oxidase, Physiol Plant 116: 135- 143.
28. - W Jarmuszkiewicz, AM Wagner, MJ Wagner and L. Hryniewiecka (1997) Immunological identification of the alternative oxidase of *Acanthamoeba castellanii* mitochondria, FEBS Lett 411: 110- 114.
29. - AL Moore, G Leach, DG Whitehouse, AWM van der Bergen, AM Wagner, K Krab (1994) Control of oxidative phosphorylation in plant mitochondria: the role of non-phosphorylating pathways, Biochim Biophys Acta 1187: 145- 151.
30. - M Chaudhuri, W Ajayi and GC Hill (1998) Biochemical and molecular properties of the *Trypanosoma brucei* alternative oxidase, Mol Biochem Parasitol 95: 53- 68.
31. - JN Siedow (1995) The active site of the cyanide- resistant oxidase from plant mitochondria contains a binuclear iron center, FEBS Lett 362: 10- 14.
32. – AL Moore, MS Albury, PG Crichton and C Affourtit (2002) Function of the alternative oxidase: is it still a scavenger? Trends Plant Sci 7: 478- 481.
33.- AM Lambowitz, JR Sobourin, H Bertrand, R Nickels and L McIntosh (1989) Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria, Mol Cell Biol 9: 1362- 1364.

34. - DM Rhoads, AL Umbach, CR Sweet, AM Lennon, GS Rauch, JN Siedow (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria, J Biol Chem 273: 30750- 30756.
35. - AL Umbach and JN Siedow (1996) The reaction of the soybean cotyledon mitochondrial cyanide resistant oxidase with sulfhydryl reagents suggests that alpha-keto acid activation involves the formation of a thiohemiacetal, J Biol Chem 271: 25019-25026.
36. - R Sotthibandhu and JM Palmer (1975) The activation of non- phosphorylating electron transport by adenine nucleotides in Jerusalem-artichoke (*Helianthus tuberosus*) mitochondria, Biochem J 152: 637- 645.
37. - T Joseph-Horne, J Babij, PM Wood, D Hollomon, RB Sessions (2000) New sequence data enable modeling of the fungal alternative oxidase and explain an absence of regulation by pyruvate, FEBS Lett 481: 141- 146.
38. - W Jarmuszkiewicz, L Hryniewiecka and FE Sluse (2002) The effect of pH on the alternative oxidase activity in isolated *Acanthamoeba castellanii* mitochondria, J Bioenerg Biomembr 34: 221- 226.
39. - CR Stewart, BA Martin, L Reding and S Cerwick (1990) Respiration and alternative oxidase in corn seedling tissues during germination at different temperatures, Plant Physiol 92: 755- 760.
40. - CR Stewart, BA Martin, L Reding and S Cerwick (1990) Seedling growth, mitochondrial characteristics, and alternative respiratory capacity of corn genotypes differing in cold tolerance, Plant Physiol 92: 761- 766.
41. - AM Wagner and AC Purvis, Production of reactive oxygen species in plant mitochondria. A dual role for ubiquinone? In: Plant mitochondria: from gene to function, pág. 537-541. edited by: IM Moller, P Garderström, K Glimelius and E Glaser (1998), Leiden, The Netherlands.
42. - Y Ito, D Saisho, M Nakazono, N Tsutsumi and A Iría (1997) Transcript levels of tandem-arranged alternative oxidase genes in rice are increased by low temperature, Gene 203: 121- 129.

43. - DP Maxwell, Y Wang and L McIntosh (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells, Proc Natl Acad Sci USA 96: 8271- 8276.

44. – BJD Meeuse (1975) Thermogenic respiration in Aroids, Annu Rev Plant Physiol 26: 117- 126.

45. - J Fang and DS Beattie (2003) Alternative oxidase present in procyclic *Trypanosoma brucei* may act to lower the mitochondrial production of superoxide, Arch Biochem Biophys 414: 294- 302.

46. - T Joseph-Horne, DW Hollomon and PM Word (2001) Fungal respiration: a fusion of standard and alternative components, Biochim Biophys Acta 1504: 179- 195.

47.- C Affourtit, SP Heaney and AL Moore (2000) Mitochondrial electron transfer in the wheat pathogenic fungus *Septoria tritici*: on the role of the alternative respiratory enzymes in fungicide resistance, Biochim Biophys Acta, 1459: 291- 298.

48. - Enzymes and their functional location: enzymes of oxygen metabolism, by S Kitaoka, Y Nakano, K Miyatake and A Yokota, In: The biology of *Euglena*, edited by DE Buetow, Volume IV (1989) Academic Press, USA, pág. 91- 94.

49. - S Kanematsu and K Asada (1979) Ferric and manganic superoxide dismutases in *Euglena gracilis*, Arch Biochem Biophys 195: 535- 545.

50. - D O´Kane, V Gill, P Boyd and R Burdon (1996) Chilling, oxidative stress and antioxidant responses in *Arabidopsis thaliana* callus, Planta 198: 371-377.

51.- M Reverberi, M Picardo, A Ricelli, E Camera, C Fanelli and AA Fabbri (2001) Oxidative stress, growth factor production and budding in potato tubers during cold storage, Free Radic Res 35: 833- 841.

52. - VN Popov, AC Purvis, VP Skulachev and AM Wagner (2001) Stress-induced changes in ubiquinone concentration and alternative oxidase in plant mitochondria, Biosci Rep 21: 369- 379.

53. - G Kocsy, G Goliba and C Brunold (2001) Role of glutathione in adaptation and signaling during chilling and cold acclimation in plants, Physiol Plant 113: 158-164.

54.- L Zhang, K Onda, R Imai, R Fukuda, H Horiuchi and A Ohta (2003) Growth temperature downshift induces antioxidant response in *Saccharomyces cerevisiae*, Biochem Biophys Res Commun 307: 308- 314.

55. - CR Stewart, BA Martin, L Reding and L Cerwick (1990) Respiration and alternative oxidase in corn seedling tissues during germination at different temperatures, Plant Physiol 92: 755-760.
56. - W Jarmuszkiewicz, O Frakzyc and L Hryniewiecka (2001) Effect of growth at low temperature on alternative pathway respiration in *Acanthamoeba castellanii* mitochondria, Acta Biochim Pol 48: 729-737.
57. - S Mishra, S Srivastava, RD Tripathi, R Govindarajan, SV Kuriakose and MN Prasad (2006) Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L, Plant Physiol Biochem 44: 25-37.
58. - L Patrick (2003) Toxic metals and antoxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity, Altrn Med Rev 8: 106-128.
59. - M Watanabe, T Suzuki (2002) Involvement of reactive oxygen stress in cadmium-induced cellular damage in *Euglena gracilis*, Comp Biochem Physiol 131: 491-500.
60. - M Watanabe, K Henmi, K Ogawa, T Suzuki (2003) Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic strains of *Euglena gracilis*, Comp Biochem Physiol 134: 227-234.
61. - AP Vitoria, PJ Lea and RA Azevedo (2001) Antioxidant enzymes responses to cadmium in radish tissues, Phytochemistry 57: 701- 710.
62. - AGM Tielens, C Rotte, JJ van Hellemond, W Martin (2002) Mitochondria as we don't know them, Trends Biochem. Sci. 27: 564-572.

CAPITULO II

-JUSTIFICACION

E. gracilis puede crecer en diversas condiciones extremas (de temperatura, concentración de oxígeno o en presencia de metales pesados). Durante el estrés a metales pesados uno de los mecanismos que utiliza es la neutralización de los metales por compuestos tioles y su compartamentalización. Sin embargo, es necesario determinar cómo funciona su compleja cadena respiratoria mitocondrial, pues en presencia de cadmio puede generar EROs o puede ser inhibida severamente. Además, es importante evaluar la respuesta celular integral al determinar la respuesta antioxidante que existe en este protista. La estrategia que utilizaremos será el enfrentar a *E. gracilis* heterotrófica (cultivada en la oscuridad) a condiciones extremas. Los modelos elegidos fueron la exposición a baja temperatura, a diferentes concentraciones de oxígeno y por último la exposición a cadmio, como modelo de exposición a metales pesados.

-HIPÓTESIS

E. gracilis puede resistir condiciones que generan estrés oxidativo y que inhiben o limitan la vía clásica mediante el aumento del contenido y actividad de la AOX, la cual drena los electrones acumulados de la poza de quinonas, atenuando así la generación de EROs.

-OBJETIVOS GENERALES

En células y mitocondrias heterotróficas de *E. gracilis*:

- Determinar el efecto de la exposición a baja temperatura, a diferentes concentraciones de oxígeno y a cadmio sobre el crecimiento celular.
- Determinar el efecto de la exposición a baja temperatura, a diferentes concentraciones de oxígeno y a cadmio sobre diferentes actividades respiratorias clásicas y alternativas.
- Evaluar el grado de estrés en las células control y expuestas a baja temperatura y cadmio.
- Evaluar la concentración y la actividad de la AOX en condiciones control y de estrés (baja temperatura y cadmio).
- Determinar la respuesta antioxidante de las células en las condiciones de estrés utilizadas.

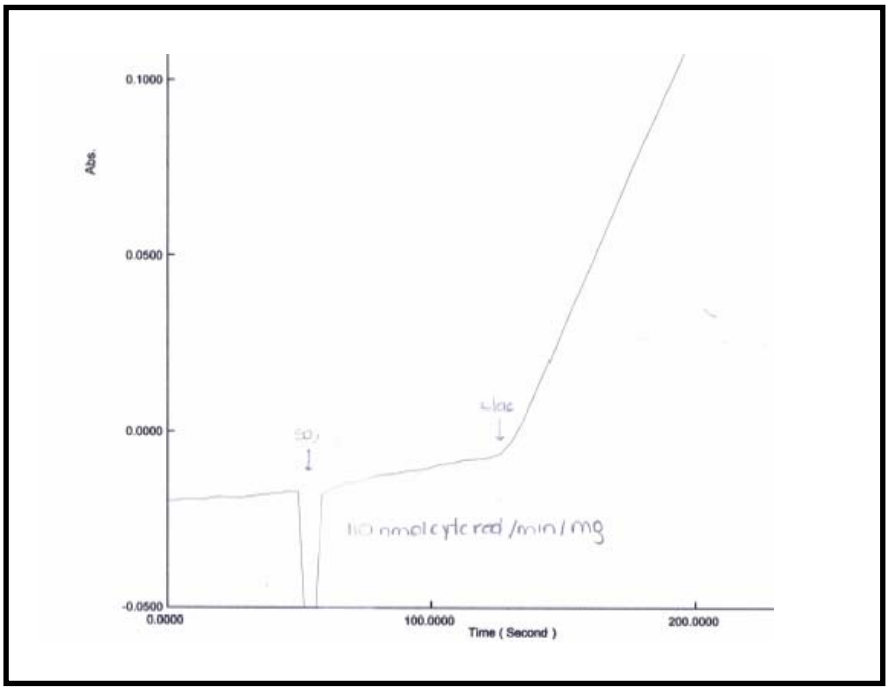
- METODOLOGIA

Oximetría concentraciones después del compuesto

La respiración fue medida como consumo de oxigeno utilizando un electrodo de oxígeno tipo Clark en un medio estándar saturado con aire que contiene KCl 120 mM, MOPS 20 mM, EGTA 1 mM (KME buffer) y fosfato 5 mM a pH de 7.3. La concentración de proteína mitocondrial utilizada fue de 1 a 3 mg. Para evaluar la capacidad de la AOX, se añadió cianuro 1 mM. La actividad correspondiente a la AOX fue sensible a 3 mM de nPG. La solubilidad del oxígeno utilizada para los cálculos fue de 533, 420 y 400 ng átomos/mL (266.5, 210 y 200 μM) para 15, 25 y 30°C, respectivamente.

Citocromo *c* reductasa

Se determinó esta actividad por espectrofotometría a 30°C utilizando 0.1 mg de proteína mitocondrial en un medio que contenía sacarosa 250 mM, HEPES 10 mM, EGTA 1 mM, ADP 1 mM y citocromo *c* de caballo oxidado 30 µM. La reacción se inició añadiendo el sustrato (10 mM de L-, D-lactato, o succinato, o 60 µM de DBQ reducido (quinona exógena)). La velocidad de reducción de citocromo *c* fue registrada como el incremento de absorbencia a 550 menos 540 nm en un espectrofotómetro dual SLM-Aminco DW2000 y en un espectrofotómetro UV-VIS Shimadzu UV-2501PC a una velocidad (slow rate) de 9000 nm/seg. El coeficiente de extinción utilizado para los cálculos fue de 19.1 mM^{-1} cm^{-1}.



Ejemplo de trazo de medición de la actividad de citocromo c reductasa.

Actividad de deshidrogenasas en mitocondrias sin quinonas

La actividad de la D-iLDH, L-iLDH y SDH en mitocondrias sin quinonas así como en la D-iLDH purificada fue medida en el amortiguador KME como el cambio de absorbencia de la UQ o RQ a 275 y 283 nm respectivamente. Los coeficientes de extinción utilizados fueron 12.5 para la UQ y 11 mM^{-1} cm^{-1} para la RQ.

Western blot

La inmunodetección fue realizada usando un anticuerpo dirigido contra la AOX de *Chlamydomonas reinhardtii*. La membrana se incubó con el primer anticuerpo a 4 °C por toda la noche, posteriormente se incubó con un anticuerpo conjugado a peroxidasa por 1 h a temperatura ambiente. La detección se realizó por quimioluminiscencia utilizando el sistema comercial ECL (Amersham Biosciences).

OTROS MÉTODOS Y PROCEDIMIENTOS EXPERIMENTALES SE DESCRIBEN DETALLADAMENTE EN LAS SECCIONES CORRESPONDIENTES DE LAS PUBLICACIONES QUE SE ANALIZAN EN LA SECCIÓN DE RESULTADOS.

CAPÍTULO III

RESULTADOS

Con resultados correspondientes al periodo de Maestría, sabíamos que la vía alterna es responsable en mayor grado de la actividad respiratoria en células estresadas con cianuro o algún otro inhibidor respiratorio clásico. Sin embargo, aunque se observó un ligero aumento de la AOX, este estrés afecto negativamente el crecimiento celular y al resto de las actividades enzimáticas mitocondriales, principalmente las que alimentan a la AOX (las deshidrogenasas).

Por esta razón decidimos examinar ahora condiciones de estrés que reflejaran una condición extrema que *E. gracilis* podría estar enfrentando fisiológicamente. Analizamos el efecto de variaciones en la temperatura, en la concentración de oxígeno y además la exposición a metales pesados usando el cadmio como modelo.

-BAJA TEMPERATURA

RESUMEN GENERAL DEL TRABAJO

Existen reportes en diferentes modelos donde la baja temperatura es causante de estrés oxidativo. Previamente, la AOX de plantas (*Petunia hybrida*, papa y soya) se ha estudiado en esta condición, mostrando un incremento en su actividad. Lo mismo se ha encontrado para la AOX de otros eucariontes como *Acanthamoeba castellani*.

Euglena gracilis fue expuesta a baja temperatura. Para obtener mayor biomasa se incubaron las células a 25°C y luego fueron transferidas a 4°C a partir de las 72 h de iniciado el cultivo. Los resultados mostraron que la exposición a baja temperatura no

afectó significativamente el crecimiento celular. Sin embargo, se encontró que sí había un efecto sobre la cadena respiratoria. La principal enzima afectada negativamente fue el complejo III. Este resultado explica la disminución de la oxidación de los sustratos a través de la vía clásica medida como consumo de oxígeno sensible a cianuro.

Por el contrario, se encontró un incremento importante en la actividad de la AOX, lo cual estuvo acompañado de un aumento en la concentración de la proteína, la cual fue detectada mediante un anticuerpo anti-AOX obtenido en contra de la enzima de *Chlamydomonas reinhardtii*.

En paralelo se analizaron algunas de las características de la AOX. Determinamos que el pH óptimo de la AOX era mas ácido que el de la citocromo *c* oxidasa, como se ha descrito también para otros organismos [39].

Este dato resulta relevante en un panorama donde la vía clásica se ve inhibida y por lo tanto la translocación de protones y la síntesis de ATP disminuyen. Esto ocasiona que el pH intramitocondrial también disminuya, favoreciendo la actividad alterna y permitiendo el continuo flujo de electrones. También observamos que la afinidad de la AOX por el oxígeno (uno de sus sustratos) fue menor que el de la citocromo *c* oxidasa en mitocondrias aisladas de células control. En contraste con lo que se ha documentado para algunos modelos donde la AOX aumenta su afinidad por oxígeno sólo en condiciones de estrés, en *Euglena* la exposición a frío, además de inducir un aumento en la afinidad por oxigeno de la AOX, produjo una disminución en la afinidad de la citocromo *c* oxidasa por este sustrato.

Estos resultados mostraron que la AOX posee un papel relevante en condiciones de estrés en mitocondrias de *E. gracilis*.

Los resultados obtenidos en esta parte del trabajo se publicaron en *Journal of Bioenergetics and Biomembranes* Vol. 36 (2004) pág. 459- 469.

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The Alternative Respiratory Pathway of *Euglena* Mitochondria

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Mitochondria, isolated from heterotrophic *Euglena gracilis*, have cyanide-resistant alternative oxidase (AOX) in their respiratory chain. Cells cultured under a variety of oxidative stress conditions (exposure to cyanide, cold, or H_2O_2) increased the AOX capacity in mitochondria and cells, although it was significant only under cold stress; AOX sensitivity to inhibitors was also increased by cold and cyanide stress. The value of AOX maximal activity reached 50% of total respiration below 20°C, whereas AOX full activity was only 10–30% of total respiration above 20°C. The optimum pH for AOX activity was 6.5 and for the cytochrome pathway was 7.3. GMP, AMP, pyruvate, or DTT did not alter AOX activity. The reduction level of the quinone pool was higher in mitochondria from cold-stressed than from control cells; furthermore, the content of reduced glutathione was lower in cold-stressed cells. Growth in the presence of an AOX inhibitor was not affected in control cells, whereas in cold-stressed cells, growth was diminished by 50%. Cyanide diminished growth in control cells by 50%, but in cold-stressed cells this inhibitor was ineffective. The data suggest that AOX activity is part of the cellular response to oxidative stress in *Euglena*.

KEY WORDS: Alternative oxidase inhibitors; cold stress; oxidative stress.

INTRODUCTION

Respiratory electron transfer in mitochondria from higher plants, fungi, and several protists is mediated by the phosphorylating cytochrome pathway and also by an alternative nonphosphorylating no-cytochrome pathway. The branching point between these two pathways is located at the ubiquinone pool level (Ito *et al*., 1997; Siedow and Umbach, 2000). The alternative pathway is resistant to antimycin and cyanide and, apparently, consists of only one enzyme, the alternative oxidase (AOX).

AOX is widespread in plants, protists, and fungi (Jarmunszkiewics *et al*., 1997; Joseph-Horne *et al*., 1998) as shown by the comparison of amino acid sequences (Day *et al*., 1995), and the cross-reactivity with an antibody raised against *Sauromatum guttatum* AOX protein (Elthon *et al*., 1989). The dimeric plant AOX is regulated by α-keto acids and by the redox-state of essential SH-groups. In contrast, fungi and protists have the active AOX as a monomeric protein regulated by purine mononucleotides (AMP or GMP) (Milani *et al*., 2001; Vanderleyden *et al*., 1980). Inhibitors of both dimeric and monomeric AOX are diphenylamine, salicylhydroxamic acid (SHAM), alkyl gallates, and disulfiram (Murphy *et al*., 1997; Siedow and Girvin, 1980; Schonbaum *et al*., 1971).

A respiratory activity resistant to antimycin and cyanide has also been described in mitochondria of the free-living protist *Euglena gracilis* (Devars *et al*., 1992; Sharpless and Butow, 1970a,b). A complex respiratory chain has been proposed for *E. gracilis*, in which not one but two additional alternative components participate in electron transfer (Moreno-Sánchez *et al*., 2000). Diphenylamine (Sharpless and Butow, 1970a), SHAM, *n*-propyl gallate (*n*PG) and disulfiram (Moreno-Sánchez *et al*., 2000) block one of these components, AOX; the other component is an antimycin-resistant quinol-cytochrome

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c oxidoreductase (Moreno-Sánchez *et al*., 2000). In regard to the entry of reduced equivalents, stereo-specific NAD^+-independent lactate dehydrogenases that donate electrons directly to the quinone pool have been described (Jasso-Chávez *et al*., 2001).

Stimulation of AOX activity by AMP is attained in mitochondria from *E. gracilis* cells cultured with succinate (Sharpless and Butow, 1970a), but not from cells cultured with glutamate plus malate as carbon source (Moreno-Sánchez *et al*., 2000; Sharpless and Butow, 1970a,b). An increase in AOX activity is achieved when cells are grown in the presence of antimycin (Sharpless and Butow, 1970a) or with ethanol as carbon source (Devars *et al*., 1998). These observations indicate that AOX in *E. gracilis* might be modulated by changing the culture conditions, i.e., by subjecting cells to a variety of stress conditions. In this regard, it is well documented that AOX activity in plants increases with cold (Stewart *et al*., 1990) or oxidative stress (Wagner, 1995).

The role of *Euglena* AOX in the physiology of the cell is unknown. Moreover, a full description of its biochemical features remains to be made. Therefore, in the present work we explored different stress culture conditions that might induce a higher AOX capacity; glutamate plus malate was used as carbon source since a higher biomass is obtained. In addition, to advance in the understanding of the AOX functioning in *E. gracilis* mitochondria, substrate dependence and inhibitor, oxygen, pH, and temperature sensitivity were also examined. The role of the second alternative component, the antimycin-resistant quinol-cytochrome *c* oxidoreductase was not explored; its participation in electron flux was merged with that of the cytochrome pathway.

MATERIAL AND METHODS

Chemicals

Salicylhydroxamic acid (SHAM), D- and L-lactic acid, *N*,*N*,*N*,*N*,tetramethyl-*p*-phenylendiamine (TMPD), disulfiram, 1,6-dichlorophenol indophenol (DCPIP), fatty acid-free bovine serum albumin, and decylbenzoquinone (DBQ) were purchased from Sigma. Sodium cyanide and sodium dithionite were from J. T. Baker; sodium azide, *n*-propyl gallate (*n*PG), and horse heart cytochrome *c* were from ICN, and diphenylamine (DPA) from Aldrich.

Cell Culture and Preparation of Mitochondria

Euglena gracilis Z, kept in the dark in liquid medium for several months, was reactivated and axenically grown in the dark with glutamate + malate as carbon source at 25 ± 2°C under orbital shaking (125 rpm) as previously described (Moreno-Sánchez and Raya, 1987). Additionally, cells were cultured for 72 h at 25°C and then either transferred at 4°C for 15 h or the medium was supplemented with H_2O_2. Cells were also cultured for 96 h at 25°C in the presence of 0.3 mM sodium cyanide added at the beginning of the culture. Cells were harvested by centrifuging at $1000 \times g$ for 10 min at 4°C and washing once in SHE medium [250 mM sucrose, 10 mM HEPES (4-(-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid), pH 7.3].

A cellular suspension of approximately 2×10^9 cells in 25 mL was sonicated as previously reported (Moreno-Sánchez and Raya, 1987) with one modification. The sonication time with a probe tip of 0.5 cm diameter was 7 s four times, with 1 min rest at 50–60% of maximal output in a Branson sonicator (Danbury, CT, USA). This modification yielded mitochondrial preparations with higher respiratory, including AOX, and dehydrogenase activities (30%, approximately). Mitochondrial protein was determined by the Biuret method as described previously (Uribe and Moreno-Sánchez, 1992).

Oxygen Uptake

The respiration of *E. gracilis* mitochondria was measured at 15–30°C, with a Clark-type oxygen electrode, in an air-saturated standard medium that contained 120 mM KCl, 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 1 mM EGTA (KME buffer), 5 mM phosphate at pH 7.3. The oxygen solubility was determined to be 533 and 400 ng atoms/mL at 15 and 30°C, respectively.

To determine the O_2 affinity of the TMPD (cytochrome *c*) oxidase, the dissolved O_2 in the KME buffer was diminished by titrating with a freshly prepared solution of dithionite. For AOX O_2 affinity, N_2-bubbled buffer was used to adjust the O_2 concentration; dithionite reaction products, particularly sulfite strongly inhibited lactate oxidation.

Dehydrogenase Activities

These were determined as previously described (Jasso-Chávez *et al*., 2001) at room temperature in 1 mL of KME buffer, 0.3 mM DCPIP, and 0.05–0.1 mg of mitochondrial protein. Succinate dehydrogenase (SDH) activity was not further increased by adding phenazine

methasulfate (0.4 mM) or Triton X-100 (0.05%), which by-pass the membrane barrier for electron transfer from the external phase.

Cytochrome *c* Reductase Activity

Mitochondria (0.1 mg protein) were incubated in 1 mL of SHE medium with 1 mM ADP and 30 μM oxidized horse heart cytochrome *c*. Adding D-lactate, L-lactate, succinate, or DBQH started the reaction. Reduction of DBQ with sodium borohydride and isolation of reduced DBQ (DBQH) with cyclopentane was made as described elsewhere (Trumpower and Edwards, 1979). The rate of cytochrome *c* reduction at 25°C was followed by the increase in the absorbance difference at 550 *minus* 540 nm in a dual wavelength SLM-Aminco DW-2000 spectrophotometer (Urbana, IL, USA); an extinction coefficient of 19.1 mM^{-1} cm^{-1} was used in the calculations (Degli Esposti and Lenaz, 1991).

Quinone Content

Extraction of quinones with isooctane from lyophilized mitochondria was made according to the procedure described by Ding *et al*. (1992). The quinones were separated and identified by reverse-phase HPLC according to Wagner and Wagner (1995). A Waters C18 Spherisorb S5 ODS2 analytical column (4.6 × 150 mm, Waters PSS 831913) was equilibrated with nitrogen-purged ethanol/methanol (3:2 v/v), and this mixture was used as the mobile phase. The flow rate was 0.5 mL/min. Detection of the quinones was performed at 275 nm. The chromatograms showed mainly the UQ-9 and RQ-9 peaks, indicating the almost complete oxidation of the quinones by this procedure. To determine the redox-state of the mitochondrial quinones under steady-state conditions, rapid extraction with petroleum ether was used (Wagner and Wagner, 1995). Quantification of quinones was estimated from the areas under the curve of the corresponding peaks using commercial Q_9 as standard. For rhodoquinone-9 identification, thin layer chromatography (TLC) and infrared spectroscopy were also used.

Cytochrome Content

Mitochondria were firstly incubated in 50% (v/v) glycerol, 60 mM KCl, 10 mM MOPS, and 0.25 mM EGTA, pH 7.3 for 30 min at 25°C under orbital shaking to induce full oxidation of endogenous substrates. The baseline (oxidized *minus* oxidized) was recorded after the mitochondrial samples were further agitated and kept under 100% O_2 gassing during the acquisition of the spectrum. The content of $c + c_1$ cytochromes was determined from ascorbate-reduced *minus* oxidized spectra; *b*-type cytochrome was estimated from the dithionite-reduced *minus* ascorbate-reduced spectra; and cytochrome $a + a_3$ from dithionite-reduced *minus* oxidized spectra. Spectra were taken in an SLM-Aminco DW2000 spectrophotometer with the bandwidth set at 2 nm and a scanning speed of 2 nm s^{-1}. The content of cytochromes $a + a_3$, b, and $c + c_1$ was estimated by using the extinction coefficients of 16 mM^{-1} cm^{-1}, 25.6 mM^{-1} cm^{-1}, and 17.5 mM^{-1} cm^{-1}, respectively (Degli Esposti and Lenaz, 1991; Gray *et al*., 1994; Priest and Hajduk, 1992).

Fatty Acids and Glutathione Content

Extraction, trans-esterification of phospholipids and derivatization of total fatty acids to methyl-esters was made as described elsewhere (Jasso-Chávez *et al*., 2002). The content of reduced glutathione in cellular extracts was determined by HPLC as previously described (Mendoza-Cózatl *et al*., 2002).

RESULTS

Growth Under Stress Conditions

Culture of *E. gracilis* in the presence of 0.3 mM cyanide diminished the rate of cell growth and lowered the cell density reached in the stationary phase (Fig. 1). The change in the incubation temperature from 25 to 4°C after 72 h of culture also lowered the growth rate and the cell density reached in the stationary phase (Fig. 1). Cells exposed to 4°C from the beginning of the culture were unable to reach the stationary phase after 7 days (data not shown). The exposure to 2 mM H_2O_2 after 72 h of incubation stopped cell growth; addition of 10 mM H_2O_2 induced cell death within the next 2 h (data not shown).

Oxidation and Dehydrogenation Rates in Isolated Mitochondria

Mitochondria isolated from cells grown in the presence of cyanide (cyanide mitochondria) showed diminished rates of L-lactate oxidation (Table I) and dehydrogenation (Table II), whereas the respective succinate-dependent reactions were unaltered. Respiratory control

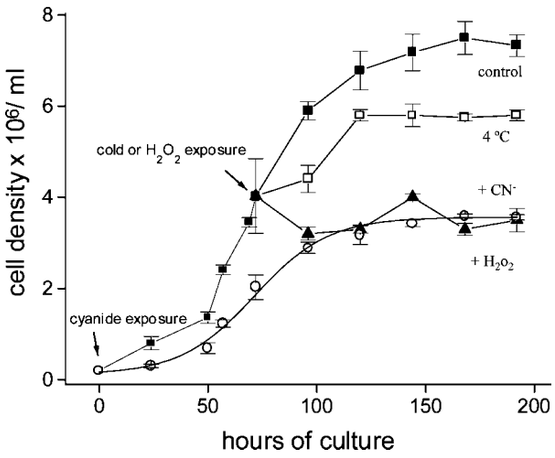

Fig. 1. Effect of stress conditions on growth of *Euglena*. The cultures were started by addition of an inoculum of 2×10^5 cells/mL in a control medium without cyanide (■) or with 0.3 mM cyanide (○). Cold (□) and 2 mM H_2O_2 (▲) stress were undertaken on control cells after 72 h of incubation. Viability was higher than 95% in all cultures.

values (rate of ADP-stimulated respiration/rate of basal respiration) were similar (1.6–1.8) in all types of mitochondrial preparations with L-lactate as substrate. With D-lactate, the rate of dehydrogenation also diminished in cyanide mitochondria, although the rate of oxidation was not affected. In mitochondria from cold-exposed cells (cold mitochondria) only L-lactate oxidation diminished, whereas succinate and TMPD oxidases, and D-iLDH and SDH activities increased. Mitochondria from H_2O_2-exposed cells (peroxide mitochondria) showed lower rates of L- and D-lactate oxidation, but similar rates of dehydrogenation, to those attained in control mitochondria; TMPD oxidase and SDH activities also decreased in peroxide mitochondria. The activity of cytochrome bc_1 complex measured directly with an artificial quinone (DBQH) (Table II) or associated with substrate dehydrogenation (not shown) also diminished in the three types of stressed-mitochondria. It should be noted that both SDH and cytochrome bc_1 activities were lower than the corresponding oxidases, indicating a suboptimal estimate of these enzyme activities. In turn, the iLDH activities fully accounted for the measured oxidase activities.

The rate of cyanide-resistant respiration in control mitochondria amounted to only 13% of total respiration with L-lactate (Table I) and was similar to previously reported data (Moreno-Sánchez *et al*., 2000). In percentage values the cyanide-resistant respiration was higher with succinate. However, it should be noted that the absolute values of cyanide-resistant respiration with either substrate were rather similar. Cyanide-resistant respiration in the other types of mitochondria followed a similar pattern to that observed in control mitochondria, except for peroxide mitochondria in which D-lactate and succinate-supported cyanide-resistant respiration were severely diminished (Table I).

Interestingly, culturing under cyanide, peroxide, or cold stress induced an enhanced cyanide-resistant respiration in isolated mitochondria with L-lactate as substrate, reaching a value of 29–34% of total respiration (Table I). In absolute rates, a significantly higher AOX capacity was attained in cold mitochondria with L-lactate or succinate.

Table I. Substrate Oxidation in *Euglena gracilis* Mitochondria

Substrate	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Peroxide mitochondria
		ng atoms oxygen min^{-1} (mg protein)$^{-1}$		
L-lactate	215 ± 15 (3)	134 ± 9 (3)[a]	174 ± 33 (8)[b]	109 ± 12 (3)[a]
D-lactate	260 ± 16 (3)	253 ± 6 (3) ns	297 ± 56 (8) ns	215 ± 24 (3)[b]
Succinate	63 ± 5 (3)	52 (2)	126 ± 30 (8)[a]	69 ± 7 (3) ns
TMPD(ascorbate	410 ± 73 (4)	264 ± 98 (3) ns	566 ± 85 (3)[b]	238 ± 22 (3)[c]
L-lactate + CN^-	28 ± 16 (3)	39 ± 10 (3) ns	50 ± 14 (8)[d]	38 ± 4 (3) ns
D-lactate + CN^-	43 ± 10 (3)	25 ± 6 (3)[b]	44 ± 15 (8) ns	19 ± 3 (3)[c]
Succinate + CN^-	34 ± 8 (3)	23 ± 8 (3) ns	58 ± 19 (6)[b]	11 ± 3 (3)[a]
TMPD-Asc + CN^-	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)

Note. Values are mean ± SD. ns = not significantly different from control. Student *t* test for nonpaired samples. (1–2 mg protein -mL) isolated from control, 0.3 mM NaCN, 4°C or 2 mM H_2O_2 exposed were incubated in KME buffer +5 mM potassium phosphate of pH 7.3 at 30°C. L-lactate, D-lactate, and succinate were added at a final concentration of 10 mM, TMPD was 2 mM (+5 mM ascorbate, Asc). 1 mM NaCN was added after 4-min incubation.

[a] $P < 0.005$ versus control.
[b] $P < 0.05$ versus control.
[c] $P < 0.025$ versus control.
[d] $P < 0.01$ versus control.

Table II. Dehydrogenase and Cytochrome *c* Reductase Activities in *E. gracilis* Mitochondria

	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Peroxide mitochondria
	nmol DCPIP red min^{-1} (mg protein)$^{-1}$			
L-$_i$LDH	258 ± 70 (5)	102 ± 40 (3)[a]	353 ± 86 (5) ns	236 ± 74 (3) ns
D-$_i$LDH	407 ± 117 (4)	209 ± 85 (3)[b]	705 ± 251 (5)[b]	385 ± 81 (3) ns
SDH	33 ± 14 (4)	27 ± 13 (3) ns	113 ± 18 (5)[c]	11 ± 3 (3)[b]
	nmol cyt *c* red min^{-1} (mg protein)$^{-1}$			
Cyt *c* reductase	96 ± 9 (5)	61 ± 7 (3)[c]	50 (2)	52 (2)

Note. Values are mean ± SD. ns = not significantly different from control. Mitochondria +0.1 mg protein /mL) were incubated at 25°C with KME buffer plus 0.3 mM DCPIP. The rates of L-$_i$LDH, D-$_i$LDH, and SDH were determined after addition of 10 mM L-lactate, 10 mM D-lactate, or 10 mM succinate, respectively. The rate of cytochrome *c* reductase was started by addition of 10 mM L-lactate in the presence of 30μM oxidized cytochrome *c*.
[a] $P < 0.025$ versus control.
[b] $P < 0.05$ versus control.
[c] $P < 0.005$ versus control.

It should be noted that inhibition of the cytochrome pathway with cyanide to fully reveal the AOX pathway does not allow for determination of the contribution to total respiration of either pathway, since inhibition of one pathway promotes the activity of the other (Day *et al*., 1996). Culturing for 40 h at 4°C yielded similar results to those achieved with 15 h of cold exposure.

In agreement with data from isolated mitochondria, AOX capacity was 26 (n = 2) and 62 ± 13 (n = 3) ng atoms oxygen (10^7 cells)$^{-1}$ min^{-1} in intact control and cold-stressed cells, respectively. After correcting for the O_2 uptake remaining in the presence of 3 mM *n*PG (and 1 mM cyanide), the AOX capacity in control and cold-stressed cells was 18.5 and 32 ± 3% of total cellular respiration, respectively. Moreover, the rate of cyanide-resistant quinol (50 μM Q_1) oxidase was 21.6 ± 2 (n = 5) and 33 ± 2 ng atoms oxygen (mg protein)$^{-1}$ min^{-1} (n = 5) in control and cold mitochondria at 30°C, respectively. At lower temperature (15°C), the cyanide-resistant Q_1 oxidation rates were 27 (2) and 46.6 (2) ng atoms oxygen (mg protein)$^{-1}$ min^{-1} in control and cold mitochondria, respectively. Thus, Q_1-dependent AOX capacity was 34 and 45% at 30°C, and 40 and 52% at 15°C of total quinol oxidase. The significant difference between these values ($P < 0.005$) suggested an increased AOX activity under cold stress.

Kinetic Properties of AOX

The temperature dependence of the respiratory activity showed that the AOX capacity remained constant throughout the range studied, whereas the cytochrome pathway diminished drastically with several substrates (Fig. 2(A)). In consequence, the contribution of each pathway to the total electron flux, in control mitochondria, is expected to vary in opposite directions with the lowering in the temperature; at 15°C both pathways showed identical maximal rates (Fig. 2(B)). In mitochondria from cold-stressed cells, the AOX pathway would appear to predominate from 10 to 20°C (Fig. 2(B)). The rate of total respiration decreased by 74, 71, and 27% at 15°C, in comparison to 30°C, in control, cyanide, and cold mitochondria, respectively (Table III). However, the rate of cyanide-resistant respiration at 15°C was similar to that attained at 30°C in control and cold mitochondria (Table III), suggesting an enhanced AOX contribution to total respiration at low temperatures.

The pH dependence of the respiratory activity at 30°C showed that the AOX capacity exhibited a maximum at a pH value of 6.55, whereas the cytochrome oxidase pathway maximum was 7.3 (Fig. 2(C)). Essentially identical pH optimal values were attained at 15°C for AOX and cytochrome pathway (not shown). These data are similar to those reported for *Acantamoeba castellani* mitochondria (Jarmuszkiewicz *et al*., 2002) in which the maximal respiratory activities were attained at pH values of 6.8 and 7.4 for the alternative and cytochrome pathway, respectively.

The TMPD oxidase exhibited a 20-fold higher affinity for oxygen than AOX in control mitochondria (Fig. 2(D); Table IV). Cold-stress slightly increased the AOX affinity for O_2 and induced a significant increase in the K_m value for oxygen of TMPD oxidase (Table IV). The K_m values for O_2 in control mitochondria were in the same range of values reported for both pathways in plant mitochondria (Ribas-Carbo *et al*., 1994; Sluse and Jarmuszkiewicz, 1998).

Titration with different AOX inhibitors showed full blockade of cyanide-resistant respiration at 15 and 30°C. The sensitivity of AOX activity towards SHAM and *n*PG was much lower in control *Euglena* mitochondria than in

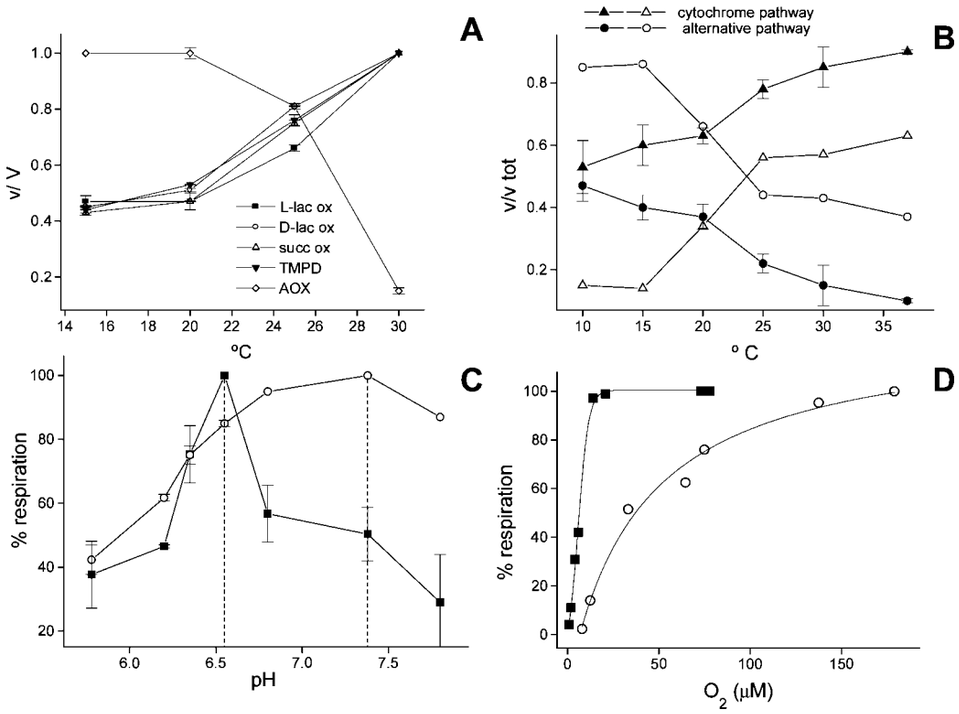

Fig. 2. Temperature, pH, and O_2 concentration dependence of the cytochrome and alternative respiratory pathways. Mitochondria (2 mg protein/mL) isolated from control (A, B) and cold-stressed cells (B) were incubated in KME buffer + 5 mM potassium phosphate, pH 7.3. After 2 min, 10 mM L-lactate (■), 10 mM D-lactate (○), 10 mM succinate (△), or 2 mM TMPD (+ 5 mM ascorbate) (▼) were added. NaCN (1 mM) was added 4 min later to mitochondria respiring with L-lactate (◇) to determine the AOX capacity. (B) The rates of respiration (v) of control (closed symbols) and cold mitochondria (open symbols) were normalized with respect to the maximal rates attained at 30°C (V, whose values are shown in Table I). (C) Control mitochondria were also incubated at 30°C at the indicated pH values. The incubation medium was as described above except that 20 mM MOPS was replaced by 10 mM MES + 10 mM HEPES + 10 mM MOPS + 10 mM Tris. The oxidizable substrate was 10 mM L-lactate and 1 mM NaCN was used to determine the AOX capacity (■). The rate of cyanide-resistant respiration was 41 ± 5 at pH 6.55. The rate of the cytochrome pathway (○) was estimated from the total rate of respiration *minus* the rate of cyanide-resistant respiration. (D) Incubation medium of pH 7.2 and thermo-stated at 30°C was either titrated with sodium dithionite (TMPD oxidase, ■), or flushed with a N_2 stream (AOX, ○), to diminish the concentration of soluble O_2. Mitochondria were then added to these media with different O_2 concentration which also contained 2 mM TMPD + 5 mM ascorbate or 10 mM L-lactate + 1 mM cyanide (AOX); the solid lines represent the best fit to the Michaelis–Menten equation.

Table III. L-Lactate Oxidation at 15°C

Substrate	Control mitochondria	Cyanide mitochondria	Cold mitochondria
	ng atoms oxygen min^{-1}(mg protein)$^{-1}$		
L-lactate	55.7 ± 8.1 (4)	38.5 ± 3.6 (3)[a]	127.4 ± 15.2 (3)[b]
L-lactate + CN^-	24.1 ± 13 (4)	20 ± 4.4 (3) ns	53 ± 11.2 (3)[a]

Note. Values are mean ± SD. ns = not significantly different from control. Student t test for nonpaired samples. Mitochondria (1-2 mg protein /mL) isolated from control, 0.3 mM NaCN or 4°C exposed were incubated in KME buffer +5 mM potassium phosphate at 15°C. L-lactate was added at a final concentration of 10 mM. 1 mM NaCN was added after 4-min incubation.
[a] $P < 0.025$ versus control.
[b] $P < 0.005$ versus control.

Table IV. AOX Affinity to O_2 and Sensitivity to Inhibitors

Inhibitor	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Reference
		$K_{0.5}$ for oxygen (μM)		
TMPDox	6.1 ± 2.6 (3)	140 ± 37 (3)[a]	90 ± 50 (4)[a]	
AOX	92.5 ± 14.8 (3)	nd	38.7 ± 1.1 (3)	
		IC_{50} (mM)		
SHAM				
15°C	0.2 ± 0.05 (3)	0.27 ± 0.06 (3)	0.45 (1)	
30°C	2.1 ± 0.3	0.29 (2)	0.3 ± 0.06 (4)[a]	0.26[b]
*n*PG				
15°C	0.5 ± 0.1 (3)	0.2 (2)	0.56 (1)	
30°C	0.8 ± 0.1	0.23 ± 0.05 (3)[a]	0.05 ± 0.008 (3)[a]	0.005[c]

Note. Values are mean ± SD. ns = not significantly different from control. The K_m for oxygen of TMPD oxidase and AOX was estimated from plots such as that shown in Fig. 3(D). IC_{50}, inhibitor concentration required to reach 50% inhibition of cyanide-resistant respiration, was measured as indicated in Fig. 4. Rates of AOX respiration are in Table I.

[a] $P < 0.005$ versus control.

[b] Skunk cabbage mitochondria[12].

[c] Mung bean mitochondria[11].

plant (Schonbaum *et al*., 1971; Siedow and Girvin, 1980), *Plasmodium* (Murphy *et al*., 1997), or in cyanide- and cold *Euglena* mitochondria (Table IV).

Stimulation of AOX activity by AMP, ADP, or GMP has been reported for several organisms (Milani *et al*., 2001; Vanderleyden *et al*., 1980). In *Euglena* mitochondria isolated from cells grown with succinate as carbon source, stimulation of succinate oxidase by AMP has been described (Sharpless and Butow, 1970a). However, in mitochondria isolated from cells grown with glutamate-malate, the AMP stimulation is negligible (Sharpless and Butow, 1970a; Moreno-Sánchez *et al*., 2000). AMP, ADP, GMP (0.5– 5 mM), as well as pyruvate (5 mM), or pretreatment with DTT (1 mM) did not affect the AOX activity in control, cyanide- and cold-mitochondria (data not shown).

Quinone, Cytochrome, Fatty Acid, and AOX Content

The HPLC analysis of mitochondrial extracts (Fig. 3(A)) revealed the presence of ubiquinone-9 (UQ-9) and rhodoquinone-9 (RQ-9), in agreement with previous reports (Powls and Hemming, 1966; Threlfall, 1972). Growth under stress conditions prompted an increase in the content of both quinones, being higher under cyanide stress (Table V). In plant tissues stored at 4°C, an increase in the total mitochondrial ubiquinone content is also found (Wagner and Purvis, 1998). Although the RQ-9/UQ-9 ratio varied from 1.3 in control mitochondria to 1.92 and 0.75 in cyanide- and cold-mitochondria, respectively, a similar lack of correlation between the rate of AOX and the contents of either UQ-9 or RQ-9 was observed (Fig. 3(B)).

The percentage reduction of the UQ-9 pool was 24.5 and 37% for control- and cold-mitochondria respiring at 30°C with L-lactate, respectively. Variation in the redox-state of the RQ-9 pool was not explored. Addition of cyanide + *n*PG prompted full UQ-9 reduction (>90%) in both types of mitochondria. Addition of cyanide alone induced full reduction of the ubiquinone pool in control mitochondria, whereas in cold mitochondria reduction was only 60.5%. In contrast, addition of *n*PG promoted 43 and 90% ubiquinone reduction in control and cold mitochondria, respectively.

Evaluation of cytochrome content in the different mitochondrial preparations showed that *a*-type cytochrome in cyanide-exposed cells diminished from 122 in control to 84 pmol (mg protein)$^{-1}$. No differences were observed between control and cold mitochondria, which were in agreement with the TMPD oxidase activities (see Table I). There were no other differences between control and cyanide- or cold-exposed cells: *b*-type and $c + c1$ cytochrome contents were 42–58 and 86–100 pmol (mg protein)$^{-1}$ for control, cyanide, and cold mitochondria.

The analysis of the fatty acid composition of the mitochondrial membranes revealed that there was a significant increase in total fatty acids induced by the exposure to stress conditions (Table VI). All the fatty acid species increased, being the polyunsaturated fatty acid (PUFA) fraction the most abundant. The unusual fatty acid $\Delta^{7,10}$ hexadecenoic acid (C16:2n-6) found in a significant amount (3–10% of total) in the plasma membranes of *Euglena*

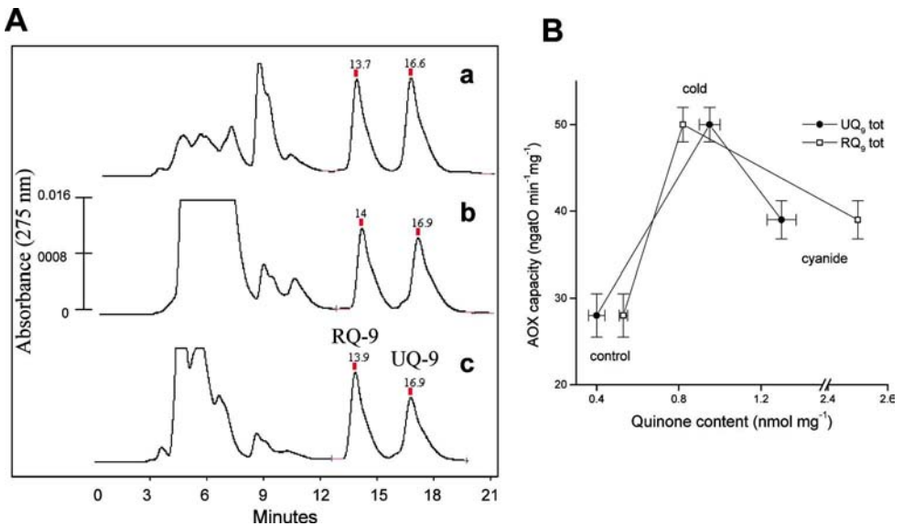

Fig. 3. (A) Quinone HPLC representative profile of *E. gracilis* mitochondria. Extraction of quinones from mitochondria (50 mg protein) was made as described in the Methods section. Mitochondria were isolated from control (a), subjected to cold stress (b), and cyanide exposed cells (c). The extract samples were 1:10, 1:20, and 1:50 diluted, respectively, and an aliquot of 50 μL was applied to the HPLC apparatus. (B) Relationship between ubiquinone and rhodoquinone content with AOX capacity in control, cyanide, and cold mitochondria. The oxidizable substrate was L-lactate. The data are presented as mean ± SD, except for the values in the *y*-axis, which represent mean ± SE for clarity purposes.

(Jasso-Chávez *et al*., 2002) was not detected in the mitochondrial membranes.

Western blot analysis of *Euglena* mitochondria with antibodies raised against AOX from either *Sauromatum guttatum* (Elthon *et al*., 1989) or *Chlamydomonas reinhardtii* (Nakamoto, 2001) revealed the presence of an increased content of a 38 kDa protein, the putative AOX, in cold mitochondria (not shown).

Physiological Role of AOX

To assess the physiological relevance of an enhanced AOX capacity, cells grown under stress conditions were further exposed to additional stimuli. Thus, cells exposed to cold stress (see Fig. 1) were harvested and further cultured in a medium that contained cyanide. The cellular density reached after 150–200 h (stationary phase) was significantly higher than that attained by control cells grown with cyanide (Fig. 4(A)). In another set of experiments, addition of *n*PG to the culture medium slightly modified the cellular density reached by control cells (approximately 15% inhibition between 120 and 200 h of culture). In contrast, *n*PG induced a marked (45%) diminution in the cellular density achieved by cold-stressed cells (Fig. 4(B)). The glutathione content in control and cold-exposed cells after 96 h culture was 4 ± 1 and 1.46 ± 0.2 nmol/(1×10^7 cells) (n = 3), respectively; the cysteine content was similar in both cellular types (0.3 nmol/(1×10^7cells).

Table V. Quinone Content in *Euglena* Mitochondria

	Control mitochondria	Cyanide mitochondria	Cold mitochondria
nmol (mg protein)$^{-1}$			
RQ_9	0.53 ± 0.02(3)	2.5 ± 0.1 (3)[a]	0.82 ± 0.2 (3)[b]
Q_9	0.4 ± 0.04 (3)	1.3 ± 0.07 (3)[a]	0.95 ± 0.05 (3)[a]

Note. Values are mean ± SD. Mitochondrial extracts (50 mg protein) were subjected to HPLC as shown in Fig. 2. Commercial Q_9 was used as standard.
[a] $P < 0.005$ versus control.
[b] $P < 0.05$ versus control.

DISCUSSION

Kinetic Properties of AOX

The *Euglena* AOX sensitivity towards three different specific inhibitors, which was similar to that observed in plant and *Plasmodium* AOX, suggests a close structural resemblance with AOX from taxonomically diverse species, at least in the binding site domain. However, the

Table VI. Fatty Acid Composition of *Euglena* Mitochondrial Membranes

	Content (μg/mg protein)		
Fatty acid	Control mitochondria	Cyanide mitochondria	Cold mitochondria
C16:0	4.3 ± 0.8	10.8 ± 3.8	14.7 ± 5.6
C16:1n-7	0.9 ± 0.3	1.6 ± 0.3	3 ± 1.5
C18:0	1.1 ± 0.6	3 ± 2.2	3.4 ± 0.6
C18:1n-9	1.5 ± 0.6	2 ± 1.7	5.7 ± 2.6
C18:2n-6	0.6 ± 0.4	1.1 ± 0.7	2.5 ± 1.7
C18:3n-6	0.4 ± 0.1(7)	0.93 ± 0.1	3 ± 1.2 (6)
C18:2n-3	0.4 ± 0.1	1.2 ± 0.6	1.6 ± 0.5
C20:0	0.3 ± 0.1 (6)	0.93 ± 0.06	1.6 ± 0.5 (6)
C20:1n-9	0.3 ± 0.1 (7)	0.6 ± 0.2	0.8 ± 0.2 (6)
C20:2n-6	1.3 ± 0.4	3.6 ± 1.6	3.6 ± 1.8
C20:3n-6	0.9 ± 0.2	3 ± 0.6	3.4 ± 1.3
C20:4n-6	7.1 ± 1.5	13.7 ± 2	17.8 ± 5.6
C20:5n-3	5.5 ± 2.1	15.7 ± 4	13.5 ± 7.6
C22:4n-6	4.5 ± 2	4.5 ± 0.8	10.3 ± 6.3
C22:5n-3	0.3 ± 0.2 (5)	1.5 ± 0.2	1 ± 0.5 (8)
C22:6n-3	5.3 ± 2	12.3 ± 3	13.2 ± 7.8
SFA	5.8 ± 1.3	14.5 ± 5.4	19 ± 6.6
MUFA	2.6 ± 0.5	4.3 ± 1.5	8.3 ± 3.5
PUFA	26.5 ± 5.5	57.5 ± 9.5	68 ± 29
TOTAL	35 ± 5.5	77.8 ± 15.7	96.5± 39

Note. Values in control, cyanide, and cold mitochondria are mean ± SD of 8, 4, and 11 independent determinations, respectively, except where indicated otherwise. SFA, MUFA, and PUFA are saturated, monounsaturated, and polyunsaturated fatty acids, respectively.

lack of effect of α-keto acids, DTT, and purine nucleotide monophosphates on *Euglena* AOX activity clearly indicates significant differences in the regulatory mechanisms with respect to other reported AOX.

The current structural model of AOX proposes an integral protein tightly bound to the inner membrane (Andersson and Nordlund, 1999), in which AOX would be sensing the matrix pH. There is no reason to expect a similar pH dependence of both cytochrome and alternative pathways. Thus, variation in the medium pH, as a tool to concomitantly vary the matrix pH, showed that optimum pH of AOX activity was slightly more acidic than that of the cytochrome pathway. A similar pattern was reported for *A. castellani*, *A. italicum*, and *S. guttatum* mitochondria (Elthon *et al*., 1986; Jarmuszkiewicz *et al*., 2002). To establish a regulatory role for the matrix pH on the AOX activity from 7.5 (matrix pH value reported for respiring mammalian mitochondria in the presence of 2 mM Pi) to 6.8, it would be required to document large physiological changes in cytosolic (and hence matrix) pH. A diminution in pH may be expected when the cytochrome pathway, which contains the H^+ pumps, becomes inactive (Jarmuszkiewicz *et al*., 2002), due to cold, oxidative or respiratory inhibitor stress.

Analysis of the steady-state UQ reduction correlated well with the respiratory rate data. The basal level of UQ reduction was lower and the respiration rate higher, in control than in cold-stressed mitochondria. Control mitochondria also showed full UQ reduction with cyanide but partial reduction with *n*PG. This UQ reduction pattern agreed with the apparent prominent contribution of the cytochrome pathway and low AOX capacity in these mitochondria. In cold mitochondria, the opposite pattern was observed in which the AOX capacity reached a value of 30% of total respiration. This correlation between UQ reduction and respiration suggests that the redox-state of the UQ pool be mainly controlled by the AOX activity in stressed mitochondria.

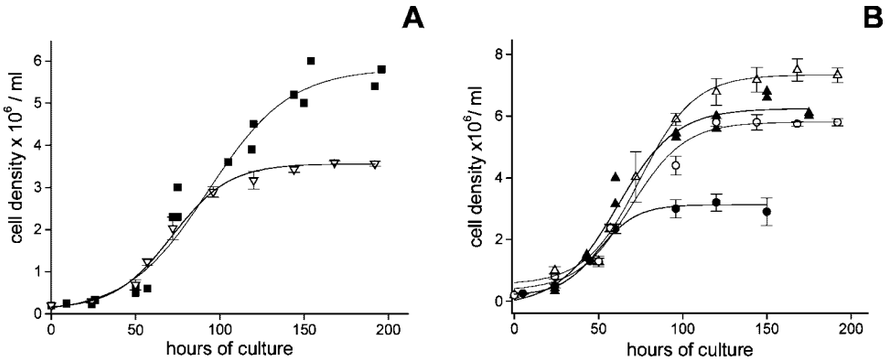

Fig. 4. AOX-dependent cellular growth. The cultures were started by addition of an inoculum of 2×10^5 cells/mL. (A) Control (∇) or cold-stressed (■) cells were cultured in the presence of 0.3 mM cyanide. (B) Control (△, ▲) or cold-stressed (○, ●) cells were cultured in the presence of 3 mM *n*PG (▲, ●).

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Rhodoquinone Pool

In *Euglena* mitochondria, RQ-9 is present in equimolar quantities to UQ-9 (Powls and Hemming, 1966; Threlfall, 1972). In this work, an increase in RQ-9 content was observed when cells were exposed to different stress conditions (cf. Table V, Fig. 3(B)). However, a role for RQ-9 in oxygen uptake has not been described. Fumarate reductase uses rhodoquinol under anoxic conditions in some eukaryotic microorganisms (Van Hellemond *et al*., 1995), but this has not been evaluated in mitochondria from *E. gracilis* grown either under aerobic or anaerobic conditions. Then, the role of RQ-9 in *E. gracilis* mitochondria, particularly under stress conditions where its content increases, remains to be explored.

Physiological Role of AOX

Mitochondria, isolated from *Euglena* cells subjected to different types of stress during culturing, showed a diminished cytochrome pathway activity and an increased capacity of the alternative respiratory pathway. The increased AOX as induced by cold stress could result from the observed change in either the quinone pool, in the fatty acid composition, or in the protein content.

Several physiological roles have been assigned to the mitochondrial AOX: (a) in plant tissues with a high AOX activity, a thermogenic effect has been proposed. The elevation in temperature would bring about volatilization of aromatic compounds to attract pollination insects (Meeuse, 1975). (b) AOX functions as an overflow valve for high levels of oxidable substrates or when the cytochrome pathway has been blocked. The electron flow provided by AOX avoids Krebs cycle complete inhibition and allows anaplerotic reactions to maintain biosynthetic pathways (Palmer, 1976). (c) AOX activity is part of the defense cellular mechanism against oxidative stress by impeding the accumulation of reduced UQ, which in turn favors the formation of reactive oxygen species (Wagner and Moore, 1997). The cyanide-resistant oxidase of *Pseudomonas aeruginosa* seems involved in cell division, growth, temperature-sensitivity, antibiotic-resistance, and oxidative stress (Reza Tavankar *et al*., 2003).

It should be noted that the three types of stress conditions used in the present work may promote accumulation of reactive oxygen species (Popov *et al*., 2001). Thus, the relative increase in AOX activity in mitochondria isolated from stressed cells suggests a role for this enzyme in the mechanisms involved in the management of oxidative stress. Furthermore, the lowering in the incubation temperature of isolated mitochondria also increased the AOX capacity, which was accompanied by a lowering of the activity of the cytochome pathway (a condition that brings about oxidative stress).

Growth of control cells was insensitive to the addition of AOX inhibitors to the culture medium. On the other hand, growth of stressed cells was highly sensitive to AOX inhibitors and resistant to cyanide. Thus, these data suggest an AOX protective role for cellular growth under stress conditions.

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REFERENCES

Andersson, M. E., and Nordlund, P. (1999). *FEBS Lett*. **449**, 17–22.
Day, D. A., Krab, K., Lambers, H., Moore, A. L., and Siedow, J. N. (1996). *Plant Physiol*. **110**, 1–2.
Day, D. A., Whelan, J., Millar, A. H., Siedow, J. N., and Wiskich, J. T. (1995). *J. Plant Physiol*. **22**, 497–509.
Degli Esposti, M., and Lenaz, G. (1991). *Arch. Biochem. Biophys*. **289**, 303–312.
Devars, S., Hernandez, R., Covián, R., García-Horsman, A., Barquera, B., and Moreno-Sanchez, R. (1998). *J. Euk. Microbiol*. **45**, 122–130.
Devars, S., Torres-Márquez, M. E., Gonzalez-Halphen, D., Uribe, A., and Moreno-Sánchez, R. (1992). *Plant Sci*. **82**, 37–46.
Ding, H., Robertson, D. E., Daldal, F., and Dutton, P. L. (1992). *Biochemistry* **31**, 3144–3158.
Elthon, T. E., Nickels, R. L., and McIntosh, L. (1989). *Plant Physiol*. **89**, 1311–1317.
Elthon, T. E., Stewart, C. E., McCoy, C. A., and Bonner, W. D., Jr. (1986). *Plant Physiol*. **80**, 378–383.
Gray, K. A., Grooms, M., Myllykallio, H., Moomaw, C., Slaughter, G., and Daldal, F. (1994). *Biochemistry* **33**, 3120–3127.
Ito, Y., Saisho, D., Nakazono, M., Tsutsumi, N., and Hirai, A. (1997). *Gene* **203**, 121–129.
Jarmuszkiewicz, W., Hryniewiecka, L., and Sluse, F. E. (2002). *J. Bioenerg. Biomembr*. **34**, 221–226.
Jarmuszkiewicz, W., Wagner, A., Wagner, M., and Hryniewiecka, L. (1997). *FEBS Lett*. **411**, 110–114.
Jasso-Chávez, R., Torres- Márquez, M. E., and Moreno-Sánchez, R. (2001). *Arch. Biochem. Biophys*. **390**, 295–303.
Jasso-Chávez, R., Vega-Segura, A., El-Hafidi, M., Moreno-Sánchez, R., and Torres.Márquez, M. E. (2002). *Arch. Biochem. Biophys.* **404**, 48–54.
Joseph-Horne, T., Wood, P. M., Wood, C. K., Moore, A. L., Headrick, J., and Hollomon, D. (1998). *J. Biol. Chem*. **273**, 11127–11133.
Meeuse, B. J. D. (1975). *Annu. Rev. Plant Physiol*. **26**, 117–126.
Mendoza-Cózatl, D., Loza-Tavera, H., and Moreno-Sánchez, R. (2002). *Physiol. Plant*. **115**, 276–283.

Milani, G., Jarmuszkiewicz, W., Sluse-Gotfart, C. M., Schreiber, A. Z., Vercesi, A. E., and Sluse, F. E. (2001). *FEBS Lett.* **508**, 231–235.
Moreno-Sánchez, R., Covián, R., Jasso-Chávez, R., Rodríguez-Enríquez, S., Pacheco-Moisés, F., and Torres-Márquez, M. E. (2000). *Biochim. Biophys. Acta* **1457**, 200–210.
Moreno-Sánchez, R., and Raya, J. C. (1987). *Plant Sci.* **48**, 151–157.
Murphy, A. D., Doeller, J. E., Hearn, B., and Lang-Unnasch, N. (1997). *Exp. Parasitol*. **87**, 112–120.
Nakamoto, S. S. (2001). *PhD Thesis in Biochemistry and Molecular Biology*, University of California at Los Angeles.
Palmer, J. M. (1976). *Annu. Rev. Plant Physiol*. **27**, 133–157.
Popov, V. N., Purvis, A. C., Skulachev, V. P., and Wagner, A. M. (2001). *Biosci. Rep.* **21**, 369–380.
Powls, R., and Hemming, F. W. (1966). *Phytochemistry* **5**, 1249–1255.
Priest, J. W., and Hajduk, J. W. (1992). *J. Biol. Chem*. **267**, 20188–20195.
Reza Tavankar, G., Mossialos, D., and Williams, H. D. (2003). *J. Biol. Chem*. **278**, 4524–4530.
Ribas-Carbo, M., Berry, J. A., Azcon-Bieto, J., and Siedow, J. N. (1994). *Biochim. Biophys. Acta* **1188**, 205–212.
Schonbaum, G. R., Bonner, W. D., Storey, B. T., and Bahr, J. T. (1971). *Plant Physiol*. **47**, 124–128.
Sharpless, T. K., and Butow, R. A. (1970a). *J. Biol. Chem*. **245**, 58–70.
Sharpless, T. K., and Butow, R. A. (1970b). *J. Biol. Chem*. **245**, 50–57.
Siedow, J. N., and Girvin, M. E. (1980). *Plant Physiol*. **65**, 669–674.
Siedow, J. N., and Umbach, A. L. (2000). *Biochim. Biophys. Acta* **1459**, 432–439.
Sluse, F. E., and Jarmuszkiewicz, W. (1998). *Braz. J. Med. Biol. Res*. **31**, 733–747.
Stewart, C. R., Martin, B. A., Reding, L., and Cerwick, S. (1990). *Plant Physiol*. **92**, 755–760.
Threlfall, D. R. (1972). *Biochim. Biophys. Acta* **280**, 472–480.
Trumpower, B. L., and Edwards, C. A. (1979). *J. Biol. Chem*. **254**, 8697–8706.
Uribe, A., and Moreno-Sánchez, R. (1992). *Plant Sci*. **86**, 21–32.
Vanderleyden, J., Peeters, C., Verachtert, H., and Bertrandt, H. (1980). *Biochem. J*. **188**, 141–144.
Van Hellemond, J. J., Klockiewicz, M., Gaasenbeek, C. P. H., Roos, M. H., and Tielens, A. G. M. (1995). *J. Biol. Chem*. **270**, 31065–31070.
Wagner, A. M. (1995). *FEBS Lett*. **368**, 339–342.
Wagner, A. M., and Moore, A. L. (1997). *Biosci. Rep.* **17**, 319–333.
Wagner, A. M., and Purvis, A. C. (1998). In *Plant Mitochondria: From Gene to Function* (Moller, I. M., Glimelius, K., and Glaser, E., eds.), Backhuys Publishers, Leiden, The Netherlands, pp. 537–541.
Wagner, A. M., and Wagner, M. J. (1995). *Plant Physiol*. **108**, 277–283.

DATOS NO MOSTRADOS DEL ARTÍCULO *Journal of Bioenergetics and Biomembranes* Vol. 36 (2004) pág. 459- 469.

(**1**) Las células expuestas a 4°C desde el inicio del cultivo no alcanzaron la fase estacionaria después de 7 días (Pág. 461).

Durante la planeación de este trabajo, se estableció el protocolo de exposición a baja temperatura después de haber transcurrido 72 h de incubación a temperatura ambiente debido a que la exposición al frío desde el inicio del cultivo mostraba un crecimiento más lento, como se muestra en la Fig. 5. Es notable que a pesar de la lentitud con la cual las células expuestas al frío desde el principio de la curva se duplican, éstas llegan a alcanzar densidades celulares similares a los cultivos expuestos en forma tardía al frío, aunque no se registró el tiempo en el cual alcanzaban su fase estacionaria.

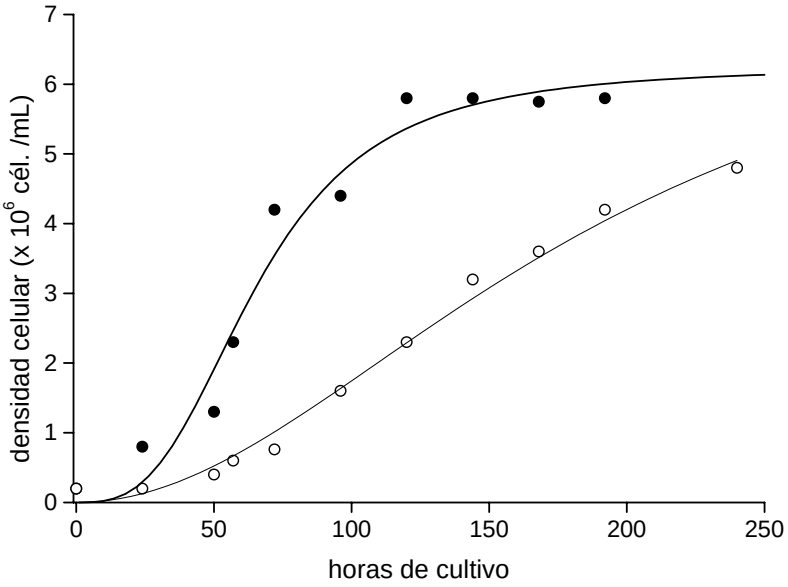

Figura 5. - Curvas de crecimiento de *E. gracilis* expuestas a 4°C. (●) células expuestas a 4°C a las 72 h de crecimiento; (○) células expuestas a 4°C desde el inicio del cultivo.

(**2**) La adición de H_2O_2 10 mM indujo muerte celular (Pág. 461).

Una de las condiciones utilizadas para generar estrés oxidativo fue la adición de peróxido de hidrógeno (H_2O_2) al medio de cultivo a las 72 h de crecimiento. Para el análisis de diferentes actividades respiratorias se utilizó la exposición a H_2O_2 2 mM. Este valor se estableció con base en estudios preliminares en donde las células se expusieron a diferentes concentraciones de este compuesto (Fig. 6), lo cual mostró que a concentraciones mayores de 2 mM las células mueren en un corto tiempo. La concentración más alta utilizada fue de 10 mM.

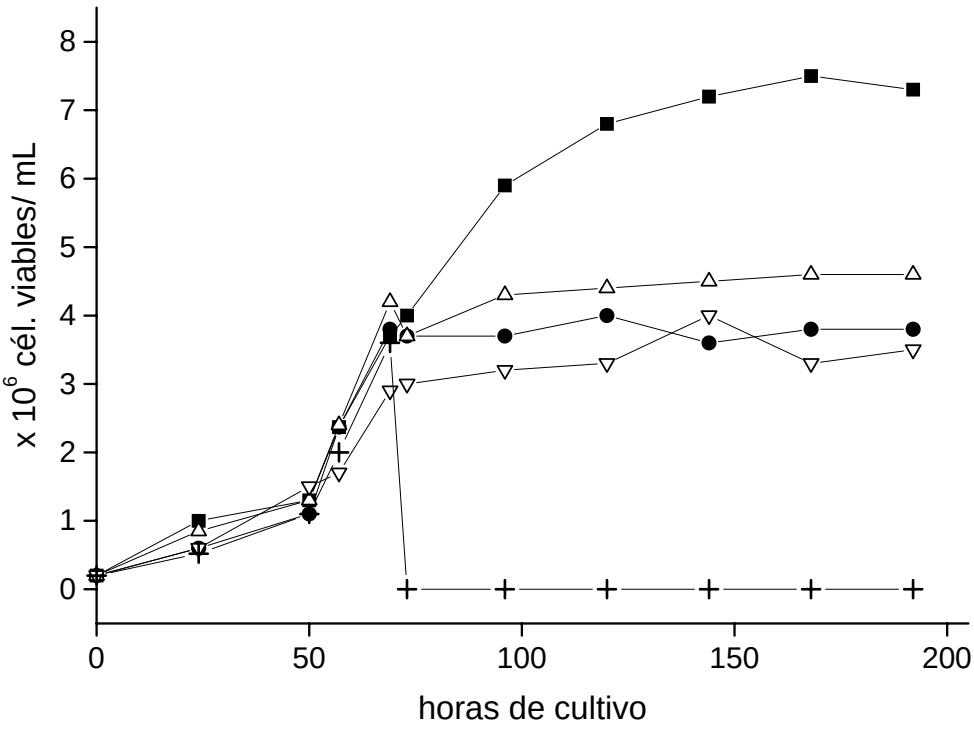

Figura 6.- Curvas de crecimiento de *E. gracilis* expuestas a H_2O_2. (■) 0 mM H_2O_2; (●) 0.1 mM H_2O_2; (Δ) 1 mM H_2O_2; (∇) 2 mM H_2O_2; (+) 10 mM H_2O_2.

(**3**) Los valores de pH óptimo obtenidos para la vía clásica y la AOX fueron similares a 15°C y 30°C (Pág. 463).

Durante la determinación de este parámetro se realizaron algunos experimentos para evaluar si existían cambios en el pH óptimo de cada vía cuando se modificaba la temperatura. Éstos se realizaron a 15°C, donde determinamos que la actividad de la AOX es mayor. Finalmente encontramos que aunque las velocidades de cada vía se modifican no había un cambio en el pH óptimo.

Tabla 2.- pH óptimo para la vía clásica y alternativa a 15 y 30°C. Las velocidades máximas obtenidas a pH óptimo de la AOX fueron 41 ± 5 y 44 ng AtO $(min\ mg)^{-1}$ respectivamente.

Temperatura	Vía clásica	AOX
30 °C	7.3	6.55
15 °C	7.3	6.4

Los resultados fueron obtenidos en un amortiguador que contenía KCl 125 mM, EGTA 1mM MOPS 20 mM MES 20 mM HEPES 20mM. La capacidad de la AOX se obtuvo en presencia de 10 mM de L-lactato y 1 mM de cianuro. La capacidad de la AOX fue sensible a 3 mM de n-PG. Los valores de pH calculados para la vía clásica fueron consistentes, ya sea cuando fueron obtenidos en presencia de n-PG o cuando se medía la actividad de TMPD oxidasa.

(**4**) AMP, ADP, GMP (0.5-5 mM) o piruvato (5mM) en mitocondrias pre-tratadas con DTT (1mM) no afectan la capacidad de la AOX en mitocondrias controles o expuestas a baja temperatura (Pág. 465). Aunque se han descrito dos diferentes tipos de regulación de las AOX en plantas y otros organismos y que existe un reporte de la activación por AMP de la AOX de *Euglena* (13), en nuestro grupo no se ha encontrado efecto alguno sobre esta actividad (14). En este trabajo utilizamos mejores condiciones para evaluar a la AOX y repetimos algunos de estos experimentos. Adicionalmente, ensayamos el efecto de otros compuestos sobre la AOX (GMP, ADP y GDP).

Los resultados se muestran en la Tabla 3:

TABLA 3.- Efecto de diferentes compuestos sobre la capacidad de la AOX

	CONTROLES	EXPUESTAS A 4°C
Capacidad de AOX	32	65
AMP 0.5 mM	31	60
1 mM	31	59
1.5 mM	31	59
GMP 0.5 mM	27.5	68
1 mM	28	68
1.5 mM	28	68
ADP 1 mM	24.5	55
GDP 1 mM	27	63
DTT 1 mM + piruvato 5 mM	26.8	52

Valores representativos medidos como consumo de oxígeno a 25 °C en amortiguador KME adicionado con fosfato 5 mM. Los trazos se realizaron con 3 mg de proteína y la capacidad de la AOX se obtuvo en presencia de 10 mM de L-lactato y 1 mM de cianuro. La capacidad de la AOX fue sensible a n-PG 3mM. Las unidades son ng At O $(min\ mg)^{-1}$.

(**5**) La concentración de AOX aumenta en células expuestas a cianuro o a 4°C (Pág. 466).

Este resultado fue obtenido mediante el uso de dos anticuerpos: uno dirigido contra *Sauromatum gutattum* y otro contra la AOX de *Chlamydomonas reinhardtii* (mostrado en la Fig. 7)

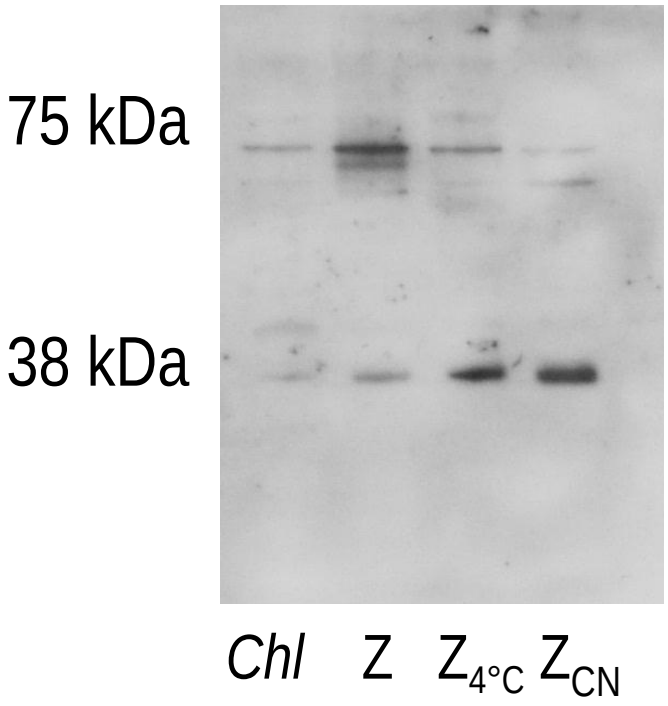

Figura 7.- Réplica en western blot de mitocondrias de *E. gracilis* controles y estresadas usando un anticuerpo contra la AOX de *C. reinhardtii*.

Estos experimentos revelaron el reconocimiento de la AOX (38 kDa) y de una proteína de mayor peso molecular y que podría corresponder a la forma dimérica. Sin embargo, la ausencia de activación por piruvato y DTT, como sucede en la enzima dimérica de plantas y por otra parte la formación de un aglomerado dimérico en la enzima de *Trypanosoma brucei* [31], la cual se sabe que funciona en su estado monomérico,

sugieren que este resultado en *E. gracilis* puede ser un artificio. Lo cierto es que hasta este momento se desconoce el mecanismo de regulación de la AOX de este protista.

- BAJA CONCENTRACIÓN DE OXÍGENO

RESUMEN DEL TRABAJO

Después de analizar la condición de estrés por temperatura abordamos otra de las condiciones planteadas: la de variaciones en la concentración de oxígeno. Para esto determinamos dos situaciones: una de concentración de oxígeno a la que las células son expuestas normalmente y otra muy baja para proceder a analizar el efecto de esta exposición sobre diferentes parámetros y actividades enzimáticas.

Además, resultados del artículo anterior mostraron que las concentraciones de rodoquinona, una benzoquinona que participa en la reducción anaeróbica del fumarato, se encuentra en concentraciones altas en condiciones aeróbicas, lo que sugiere una función relevante y novedosa. Al trabajar en condiciones que teóricamente favorecerían su función, nos abocamos a analizar y evaluar su participación en la transferencia de electrones en mitocondrias de *E. gracilis*.

Los resultados mostraron inicialmente que existe una actividad de fumarato reductasa importante en condiciones aeróbicas. *E. gracilis* puede tolerar condiciones de baja concentración de oxígeno hasta un cierto límite (aproximadamente 60 µM), pero en condiciones donde es viable suceden cambios metabólicos importantes como una disminución en la actividad respiratoria tanto clásica como de la AOX, y un aumento en el transporte de electrones a través de la reacción anaeróbica de la fumarato reductasa. Adicionalmente encontramos que la relación RQ/ UQ aumentó, como se esperaría en una

condición de baja concentración de oxígeno y la cual se ha documentado en otros organismos como el nemátodo parásito de cerdos *A. suum* [21].

De forma interesante encontramos que al utilizar como sustrato a la RQ purificada y reducida de *E. gracilis*, ésta podía participar transportando electrones a la respiración aeróbica clásica (a través de los dos complejos III y alterno tipo-III) y alternativa, aunque con menor eficiencia que usando ubiquinol.

Otra conclusión importante de este trabajo surgió al evidenciar la reducción específica de la RQ por la oxidación de D-lactato a través de la D-iLDH presente en la membrana mitocondrial y caracterizada en nuestro grupo [15].

Los resultados correspondientes a esta parte del trabajo se publicaron en *Biochimica Biophysica Acta- Bioenergetics* Vol: 1710 (2005) pág. 113-121.

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Physiological role of rhodoquinone in *Euglena gracilis* mitochondria

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Abstract

Rhodoquinone (RQ) participates in fumarate reduction under anaerobiosis in some bacteria and some primitive eukaryotes. *Euglena gracilis*, a facultative anaerobic protist, also possesses significant rhodoquinone-9 (RQ_9) content. Growth under low oxygen concentration induced a decrease in cytochromes and ubiquinone-9 (UQ_9) content, while RQ_9 and fumarate reductase (FR) activity increased. However, in cells cultured under aerobic conditions, a relatively high RQ_9 content was also attained together with significant FR activity. In addition, RQ_9 purified from *E. gracilis* mitochondria was able to trigger the activities of cytochrome bc_1 complex, bc_1-like alternative component and alternative oxidase, although with lower efficiency (higher K_m, lower V_m) than UQ_9. Moreover, purified *E. gracilis* mitochondrial NAD^+-independent D-lactate dehydrogenase (D-iLDH) showed preference for RQ_9 as electron acceptor, whereas L-iLDH and succinate dehydrogenase preferred UQ_9. These results indicated a physiological role for RQ_9 under aerobiosis and microaerophilia in *E. gracilis* mitochondria, in which RQ_9 mediates electron transfer between D-iLDH and other respiratory chain components, including FR.

Keywords: Microaerophilia; Rhodoquinone redox state; Fumarate reductase; Pyridine nucleotide-independent D-lactate dehydrogenase; Cytochrome bc_1 complex; Alternative oxidase

1. Introduction

Rhodoquinone (RQ; E°′ = −63 mV) is an essential component in fumarate reduction during anaerobic electron transfer in mitochondria of some primitive eukaryotic organisms such as *Ascaris suum* [1] and *Fasciola hepatica* [2] as well as in rhodobacteria [3]. Quinol fumarate reductases (QFR) in other types of bacteria use low potential menaquinone (E°′ = −74 mV) [4]. In *A. suum*, a correlation was observed between the RQ content and the rate of fumarate reduction in vivo. Mitochondria isolated from adult anaerobic *A. suum* diminish their cytochrome and ubiquinone (UQ) contents, whereas that of RQ substantially increases [1].

E. gracilis, a photosynthetic and facultative anaerobic protist, has a complex mitochondrial electron transfer chain constituted by typical respiratory enzymes (complexes I, II and IV) and a cytochrome bc_1 complex that is antimycin-sensitive but myxothiazol-resistant [5]. Alternative respiratory components are also present: NAD^+-independent L- and D-lactate dehydrogenases (L- and D-iLDH) [6], an alternative oxidase (AOX) [7], and bc_1-like activity (bc_1-*bypass*) sensitive to myxothiazol but antimycin-resistant [5]. Based on respiratory inhibition studies, the electron flux across the respiratory chain has been partially elucidated [5].

Rhodoquinone-9 (RQ_9) and ubiquinone-9 (UQ_9) are detected in *E. gracilis* [8]. Under aerobic growth conditions, RQ_9 is present in *E. gracilis* mitochondria with similar [7,8] or lower [9] concentration than UQ_9. On the other hand, under anoxic culture conditions the RQ_9 content increases whereas that of UQ_9 decreases in *E. gracilis* cells [9]. A significant concentration of RQ_9 is also found in mitochondria isolated from dark grown *E. gracilis*, which lacks chlorophyll and chloroplasts [7,8]. Moreover, an increase of both UQ_9 and RQ_9 is attained when cells are grown under cold or oxidative stress [7].

The last findings suggest that RQ in *E. gracilis* mitochondria might be engaged in other functions under aerobiosis, in addition to participating in fumarate reduction under anaerobiosis. Therefore, to elucidate the physiological relevance of RQ in *E. gracilis*, the effect of culturing under microaerophilic and aerobic conditions on the quinone pool and respiratory activities was analyzed.

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2. Materials and methods

2.1. Chemicals

Decylubiquinone (DBQ), ubiquinone-1 (UQ_1), ubiquinone-2 (UQ_2), ubiquinone-9 (UQ_9), DCPIP (2,6-dichlorophenol indophenol), antimycin, theonyltrifluoroacetone (TTFA), 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and methyl viologen were from Sigma Chem. Co. Rhodoquinone-9 (RQ_9) and rhodoquinone-10 (RQ_{10}) were purified from *Euglena gracilis* and *Rhodospirillum rubrum* as described below. Horse heart cytochrome *c*, n-propyl gallate (nPG) and myxothiazol were from ICN; stigmatellin was from Fluka, and isooctane from J. T. Baker.

2.2. Cell culture and preparation of mitochondria

Cells of *E. gracilis* Z were grown in the dark in the Hutner's acidic organotrophic medium with glutamate+malate as carbon source [10] at 25±1 °C under orbital shaking (125 rpm). Aerobic and microaerophilic cultures were inoculated with aerobic-cultured *E. gracilis* cells to a final concentration of 2×10^5 cells/ ml. The aerobic cultures contained 1 l of medium in 2-l Erlenmeyer flasks. Microaerophilic cultures were carried out culturing cells in 2 l Erlenmeyer flasks containing 2 l of medium previously bubbled with N_2 and then sealed and incubated without shaking.

Mitochondria were isolated as previously described [7] from cells reaching the stationary phase (96 h of culture). Mitochondrial protein was determined by the Biuret method as described elsewhere [11].

2.3. Cytochrome content

Reduced *minus* oxidized difference spectra of mitochondria was determined as previously described [7]. The content of cytochromes $a+a_3$, b and $c+c_1$ was estimated by using the extinction coefficients of $\varepsilon=16$ mM^{-1} cm^{-1} (609–630 nm) [12], $\varepsilon=25.6$ mM^{-1} cm^{-1} (563–578 nm) [13] and $\varepsilon=17.5$ mM^{-1} cm^{-1}(561–540 nm) [14], respectively.

2.4. Quinone analysis

Quinone-depleted mitochondria were obtained from lyophilized mitochondria that were incubated with isooctane for extraction of quinones according to the procedure previously described [7,15]. The quinones extracted from mitochondria were separated and identified by reverse-phase HPLC according to Wagner and Wagner [16]. Quinones were separated with a Waters C18 Spherisorb S5 ODS2 analytical column (4.6×250 mm, Waters PSS 831913) using nitrogen-purged ethanol/methanol (3:2 v/v) as the mobile phase. The flow rate was 1 ml/min. Detection of UQ and RQ was made at 275 and 283 nm, respectively. RQ_9 was purified from *E. gracilis* grown in aerobic conditions and RQ_{10} from *Rhodospirillum rubrum*. For RQ purification and identification, thin layer chromatography (TLC), UV-absorption spectrophotometry and infrared spectroscopy were used. To determine the redox state of the mitochondrial quinones under steady-state conditions, a rapid extraction with petroleum ether was used [16].

2.5. Enzyme activities

D-iLDH, L-iLDH and succinate dehydrogenase (SDH) activities in intact fresh mitochondria were determined as previously described [6] at room temperature in 1 ml of KME buffer (120 mM KCl, 20 mM MOPS (3-(N-morpholino)-propanesulfonic acid), 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), pH 7.3), 0.3 mM of DCPIP and 0.05–0.1 mg of mitochondrial protein. The reaction was started by adding L-lactate, D-lactate or succinate and the rate of DCPIP reduction was determined by measuring the absorbance change at 600 nm and using an extinction coefficient of $\varepsilon=21.3$ mM^{-1} cm^{-1} [17].

In contrast, activity of dehydrogenases in quinone-depleted mitochondria and that of purified mitochondrial D-iLDH were measured by following the change in absorbance of UQ or RQ at 275 and 283 nm, respectively. Extinction coefficients of $\varepsilon=12.5$ mM^{-1} cm^{-1} (from $E_{1\%1\ cm}=158$ at 283 nm) [18] for RQ and $\varepsilon=11$ mM^{-1} cm^{-1} (from $E_{1\%1\ cm}=141$ at 275 nm) [19] for UQ were used for calculations. The rate of quinone reduction was corrected for the remaining activity after adding 10 mM oxalate (L-iLDH, D-iLDH) or 10 mM malonate (SDH). These assays were carried out at 30 °C in 1 ml of KME buffer and with 0.015% triton X-100, when hydrophobic quinones were added; the reaction was started by adding the respective substrate. The purified D-iLDH enzyme solution contained 2.8% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 10 mM HEPES and 1 mM EGTA, pH 8 [20]. The amount of purified D-iLDH added in each assay was 50 μg protein (and 0.4% CHAPS final concentration). The purity of D-iLDH, determined by densitometric analysis of Coomassie blue stained SDS-PAGE [20], was approximately 60%. Incubation of enzyme with quinones for 2–10 min gave similar results; this indicated that the interaction of quinone-detergent micelles with the purified enzyme was relatively fast under the conditions assayed.

Fumarate reductase (FR) activity was measured at 30 °C using methyl viologen (MV) as electron donor ($\varepsilon=11.4$ mM^{-1} cm^{-1} at 600 nm) [21]. MV was reduced with dithionite until the absorbance reached a value of 0.8. The MV oxidation was monitored under a N_2 stream in the KME buffer using 0.3 mg protein; the reaction was started by adding 10 mM of freshly prepared fumarate. Non-enzymatic MV oxidation (<15%) was considered in the calculations.

Reduction of quinones with sodium borohydride and isolation of reduced quinones with cyclopentane was made as described elsewhere [22]. Reduced DBQ ($DBQH_2$), reduced UQ_9 (UQ_9H_2) and reduced RQ_9 (RQ_9H_2) (60 μM) were added to reconstitute electron transport, and oxygen uptake, in presence of triton X-100 (0.015%). A significant diminution of enzyme activities was obtained when sonication was used. Therefore quinones were incorporated into membranes by detergent solubilization.

Cytochrome *c* reductase activity was measured at 30 °C as previously reported [7] using quinone-depleted mitochondrial preparations. The reaction was started by adding 10 mM L-lactate or 60 μM $DBQH_2$, UQ_9H_2 or RQ_9H_2. Addition of 0.5 μM stigmatellin fully inhibited the reduction of cytochrome *c*. For the addition of hydrophobic quinones (UQ_9 and RQ_9), triton X-100 (0.015%) was also present. This detergent concentration yielded the highest activities; activity was negligible when reduced quinones were added in the absence of detergent. The cytochrome bc_1 complex and bc_1-*bypass* activities were estimated by adding 0.5 μM antimycin or 10 μM myxothiazol, respectively. Assays were also carried out with purified *E. gracilis* cytochrome bc_1 complex [23], using a protein concentration of 25–50 μg and incubated in 1 ml of KME buffer with 30 μM horse heart cytochrome *c* and 0.015% triton X-100, to allow solubilization of hydrophobic quinones. The activity of purified cytochrome bc_1 complex diminished (40%) by adding triton X-100, with no effect on K_m values for $DBQH_2$ and UQ_1H_2. The reaction was started by adding reduced quinone. The addition of 0.5 μM antimycin abolished the activity. An extinction coefficient for cytochrome *c* of $\varepsilon=19.1$ mM^{-1} cm^{-1} (550–540 nm) was used in the calculations [13].

The rate of respiration of fresh *E. gracilis* mitochondria was measured at 30 °C, with a Clark-type oxygen electrode, in an air-saturated KME buffer with 5 mM phosphate. Cyanide and nPG were added to evaluate the AOX capacity. TMPD oxidase activity was evaluated at 30 °C by determining the rate of O_2 uptake after adding 5 mM ascorbate plus 2.5 mM TMPD, which was fully inhibited by 10 mM azide or 1 mM cyanide. The oxygen solubility was determined to be 400 ng atoms/ml (200 μM) at 30 °C.

2.6. Determination of metabolites

L-lactate and D-lactate were determined by fluorometry at 30 °C according to standard methods [24]. Succinate was determined by measuring the amount of O_2 consumed by bovine heart or rat liver submitochondrial particles (2 mg protein) [25] at 25 °C in SHE medium (250 mM sucrose, 10 mM HEPES (4-(-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA, pH 7.3)+1 μM rotenone. This rate of O_2 uptake was fully inhibited by 5 mM malonate or 1 μM antimycin. The concentration of dissolved O_2 in the culture medium was estimated by using a Clark-type O_2 electrode.

3. Results

3.1. Microaerophilic exposure

E. gracilis can grow under different oxygen concentrations [26]. Growth of dark-adapted *E. gracilis* under microaerophilic condition ([O_2]=63–105 μM) was still significant, although a 70% decrease in biomass was observed in comparison to aerobic condition (Fig. 1). Cell growth was abolished in cultures with lower O_2 concentrations (<63 μM) (data not shown).

Mitochondria isolated from microaerophilic cells showed a marked decrease (approximately 50%) in respiratory rates with succinate, L-lactate and D-lactate. Also, generation of membrane potential by oxidation of the same substrates was not apparent in these mitochondria (data not shown), when it was determined with safranine O or rhodamine 123 as described elsewhere [27,28].

The cytochrome bc_1 complex activity diminished under microaerophilic culture conditions (Table 1), which correlated with the severe lowering in the *b*-type cytochrome content (Table 2). The activity of complex IV (TMPD oxidase) showed no variation under both conditions (Table 1), which in turn correlated with the slight diminution of *a*-type cytochrome in mitochondria from microaerophilic cultures (Table 2). The RQ_9 content slightly increased (10%) when *E. gracilis* cells were cultured under microaerophilic condition, whereas their UQ_9 content diminished by 37% (Table 2). In consequence, the RQ_9/UQ_9 ratio increased from 1.32 under aerobiosis to 2.32 under microaerophilic condition.

Fumarate reduction was detected in aerobic *E. gracilis* isolated mitochondria (Table 1). Moreover, the FR activity was markedly higher than SDH activity, which catalyzes the reverse reaction of FR. HQNO, an inhibitor of bacterial FR [29], did not significantly affect *E. gracilis* FR activity, whereas eukaryotic SDH inhibitor TTFA [4,30] induced a partial inhibition of 50% in both types of mitochondria (Table 1). Malonate (10 mM) and oxaloacetate (0.1 mM), typical SDH inhibitors [31], strongly blocked FR activity by 73 and 90%, respectively, whereas SDH activity was fully inhibited (>95%) in both types of mitochondria.

A significant decrease in dehydrogenase activity was also observed in mitochondria isolated from microaerophilic cultures (Table 1), particularly L-iLDH. Although SDH and D-iLDH showed a similar decrease, D-iLDH had the highest activity. Moreover, the D-iLDH /SDH activity ratio (7.7) was similar under aerobiosis or microaerophilia, whereas the D-

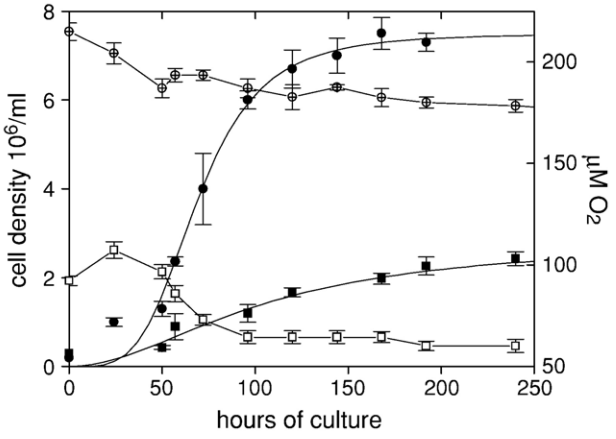


Fig. 1. Effect of the oxygen concentration on *E. gracilis* growth. The cultures were started by adding an inoculum of 2–3×10^5 cells/ ml in control, aerobic (●) or N_2-bubbled (■) medium. Viability was higher than 95% in both cultures. Values are mean±standard deviation (S.D.) of 4 different cell cultures. The oxygen concentration in the aerobic (⊕) and N_2-bubbled (□) culture media was determined with a Clark-type oxygen electrode in a culture aliquot at each indicated time. The air-saturated fresh culture medium showed an O_2 concentration of 215 μM at 25 °C. After 96 h of cell culture, the O_2 concentration decreased to a value of 170 μM. In the N_2-bubbled medium, the O_2 concentration was initially 92 μM and after 96 h of culture was 64 μM.

Table 1
Enzyme activities in *E. gracilis* mitochondria isolated from aerobic and microaerophilic cultures

Enzyme	Aerobic mitochondria	Microaerophilic mitochondria
Fumarate reductase	112±15 (4)	213±22 (4)[a]
+TTFA	56±6 (3)	99.5±6 (3)[b]
+HQNO	110±11 (3)[c]	198±22 (3)[c]
SDH	38±13 (5)	10.6±4 (7)[a]
L-iLDH	176±45 (5)	29±3 (7)[d]
D-iLDH	292±48 (5)[a]	82±19 (7)[d]
bc_1 complex	96±9 (5)[e]	39±5 (4)[d]
TMPD oxidase	410±73 (4)[e]	389±28 (4)[c]
AOX	30±3 (3)	29±4 (3)[c]
bc_1-bypass	57±12 (3)	19.4±6.7 (3)[d]

Values are mean±S.D. Fumarate reductase activity is in nmol reduced fumarate (min×mg protein)$^{-1}$; dehydrogenase activities are in nmol reduced DCPIP (min×mg protein)$^{-1}$; cytochrome bc_1 complex and *bc_1-bypass* activities are in nmol reduced cytochrome *c* (min×mg protein)$^{-1}$; TMPD oxidase activity is in nanogram atoms oxygen (min×mg protein)$^{-1}$ and AOX values are presented as percentage of total respiration with L-lactate. FR inhibitors HQNO and TTFA were added to a final concentration of 10 μM.
[a] $P<0.05$ versus aerobic mitochondria.
[b] $P<0.05$ versus microaerophilic mitochondria in absence of inhibitor.
[c] Not statistically different.
[d] $P<0.005$ versus aerobic mitochondria.
[e] Data reported in Castro-Guerrero et al. [7].

Table 2
Effect of the oxygen concentration on cytochrome and quinone content

Cytochrome or quinone content	*E. gracilis* mitochondria		*A. suum* mitochondria[a]	
	Aerobic culture	Microaerophilic culture	Aerobic culture[b]	Microaerophilic culture[c]
a	128±12 (3)	92±15 (5)[d]	150	n.d.
b	60±11 (3)	18±3 (5)[d]	440	190
$c+c_1$	89±7 (3)	80±5 (5)[e]	370	36
UQ_9	0.4±0.04 (3)[f]	0.25±0.01 (3)[g]	0.33	n.d.
RQ_9	0.53±0.02 (3)[f]	0.58±0.02 (3)[d]	0.12	1.9

Values are mean±S.D. Cytochrome content units are pmol (mg protein)$^{-1}$; quinone content units are nmol (mg protein)$^{-1}$; n.d. not detected.
[a] From results reported by Takamiya *et al*. [1].
[b] Data from *Ascaris* L2 [1].
[c] Data from *Ascaris* adult [1].
[d] $P<0.05$ versus aerobic culture (Student *t* test for non-paired samples).
[e] Not statistically different from aerobic culture.
[f] Data reported by Castro-Guerrero et al. [7].
[g] $P<0.005$ versus aerobic culture.

iLDH/L-iLDH activity ratio was 1.6 under aerobiosis and it increased to 2.8 under microaerophilic conditions. Thus, these data suggested that under microaerophilic growth conditions the mitochondrial quinone pool was predominantly reduced by D-iLDH.

A high concentration of D-lactate and L-lactate was previously reported for *Euglena* [32]. The intracellular D-lactate content in cells harvested in the stationary phase did not change with the growth condition (4.7±0.7 (n=3) and 5.8±1.2 (n=3) nmol/10^7 cells under aerobiosis and microaerophilia, respectively), whereas that of L-lactate decreased, slightly, from 16±1.7 (n=3) to 13.2±0.8 (n=3) nmol/10^7 cells. The succinate content increased from 5.8 nmol/10^7 cells in aerobiosis to 12.3 nmol/10^7 in microaerophilia. Intriguingly, the concentration of L-lactate in the culture medium after 96 h of cell growth increased from 0.08 mM (n=2) under aerobiosis to 0.79±0.07 (n=3) mM under microaerophilia. The succinate concentration in the culture medium also increased from 0.21±0.08 (n=3) mM in aerobiosis to 0.4±0.1 mM (n=3) in microaerophilia. In contrast, no change in the D-lactate concentration (0.014 mM) in the culture medium was observed.

3.2. Analysis of quinone pool reduction

To establish the relationship between the UQ_9 and RQ_9 redox states with the rate of electron transfer, respiratory rates were titrated with oxalate (for lactate oxidation) or malonate (for succinate oxidation). Inhibition of the respective dehydrogenase induced a progressive diminution in the reduction level of UQ_9 (Fig. 2A). Interestingly, for RQ_9, a similar pattern was attained solely with D-lactate oxidation, whereas with succinate or L-lactate, the redox state of RQ_9 was unaltered (Fig. 2B). With D-lactate, addition of cyanide brought the level of reduced RQ_9 up to 84% (data not shown).

3.3. Role of RQ in Euglena mitochondria

In order to analyze the interactions of RQ with other respiratory components, RQ from *E. gracilis* (RQ_9) and *R. rubrum* (RQ_{10}) were purified (Fig. 3A). The purification procedure of RQ yielded a compound with an absorption maximum at 283 nm, without apparent contamination by UQ, which has an absorption maximum at 275 nm (Fig. 3B).

To analyze the possible role of RQ in the aerobic electron transfer, the activities of several dehydrogenases and cytochrome c reductases were determined in quinone-depleted mitochondria using as substrates oxidized or reduced RQ and UQ, respectively (Tables 3 and 4). As expected, activity with UQ_9 and some analogues (DBQ, UQ_1, UQ_2) was attained for both, SDH and L-iLDH but no activity with RQ_9 or RQ_{10} was evident, in agreement with the data of Fig. 2. For D-iLDH, similar rates were achieved with both types of quinones (Table 3).

RQH_2 was able to mediate the reduction of cytochrome c in a stigmatellin-sensitive reaction (Table 4). Cytochrome bc_1 complex and bc_1-*bypass* are sensitive to stigmatellin [5]. The involvement of these two different components that reduce

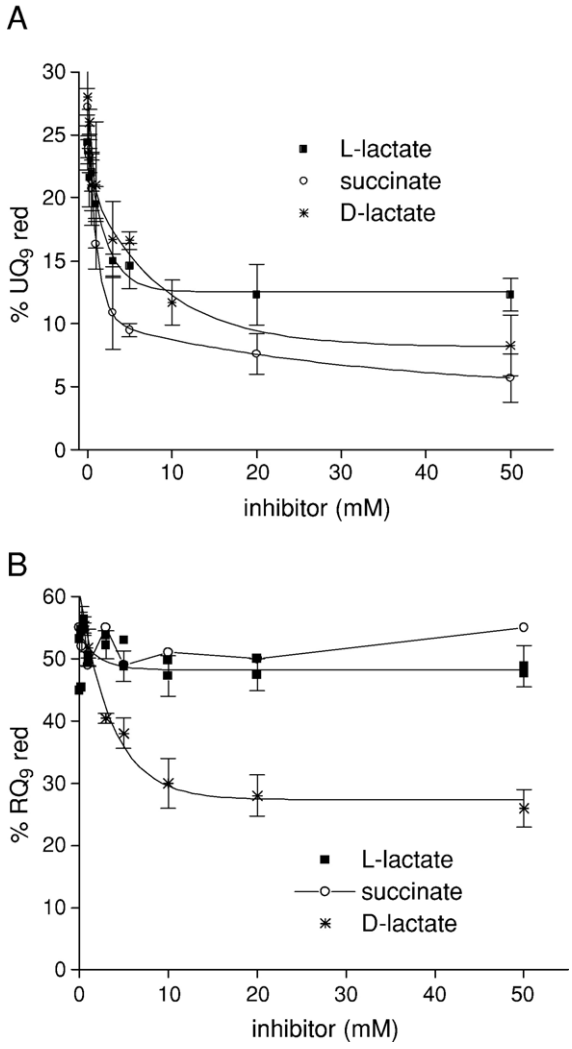


Fig. 2. Effect of substrate oxidation on redox state of quinone pool in *Euglena* mitochondria. (A) Redox state of UQ using succinate (○), L-lactate (■) or D-lactate (*) as substrate. (B) Redox state of RQ with succinate (○), L-lactate (■) or D-lactate (*) as substrate. Abbreviations: Qred=reduced quinone. Values are mean±S.D. of 3 different mitochondrial preparations except for RQ_9 red with succinate, where values represent mean of two preparations. The percentage of UQ reduction reached 90–95% when 1 mM cyanide was added to inhibit COX, whereas addition of nPG, inhibitor of the AOX, increased the quinone reduction to 43%. A non-reducible portion of UQ of 5% of the total was revealed with dithionite, whereas a 5% of the total was non-oxidizable after the samples were stirred and kept under 100% O_2 gassing.

cytochrome c in *E. gracilis* mitochondria was resolved by adding other inhibitors. Myxothiazol was added to inhibit the alternative cytochrome c reductase (bc_1-*bypass*) and antimycin to inhibit cytochrome bc_1 complex activity [5]. Inhibition by antimycin of cytochrome c reduction was similar with UQ (62%) or RQ (65%); the remaining activity was fully blocked by myxothiazol (data not shown). These data indicated that both cytochrome bc_1 complex and bc_1-*bypass* were able to use RQ as substrate, although the bc_1-*bypass* activity showed a higher K_m value for both quinones than cytochrome bc_1 complex (Table 4).

Purified *E. gracilis* D-iLDH reduced both the RQ_9 and UQ_9 pools with similar V_m values (1360 and 1160 nmol (min×mg

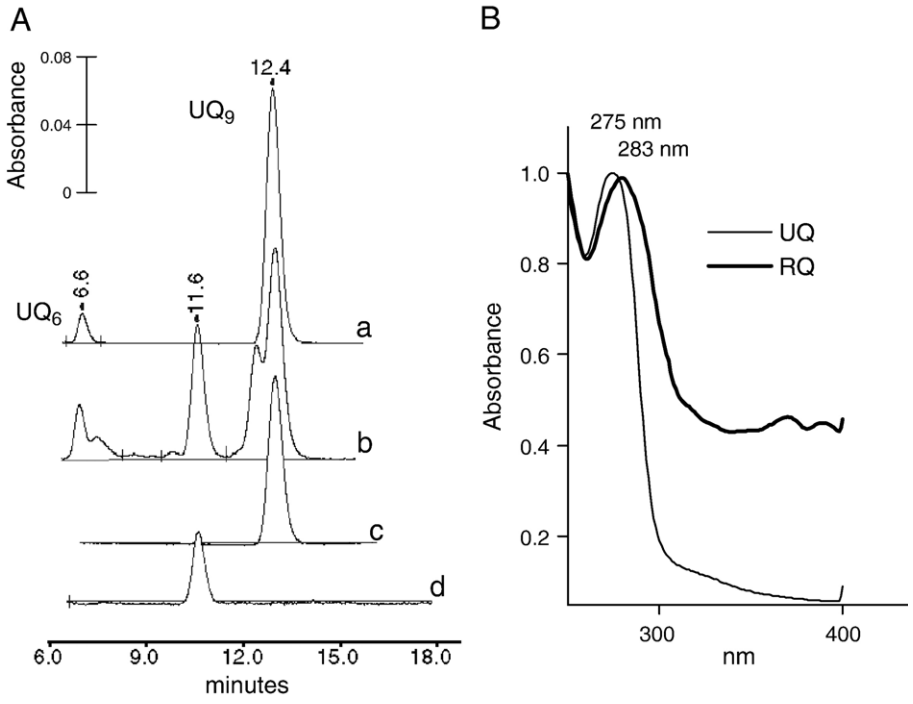


Fig. 3. Purification of rhodoquinone and ubiquinone from *Euglena* mitochondria. (A) HPLC profiles of Q_6 and Q_9 standards (a), an extract from *E. gracilis* mitochondria (b), purified UQ_9 (c) and purified RQ_9 (d) from *E. gracilis* mitochondria. (B) Absorption spectra of purified RQ and UQ from *E. gracilis*.

protein)$^{-1}$, respectively), although a lower K_m value for the former was observed (18±7 μM, (n=3), and 52±10 μM, (n=3), respectively). In turn, purified *E. gracilis* cytochrome bc_1 complex was also able to oxidize both reduced quinones, in an antimycin-sensitive reaction (Fig. 4), but in contrast to purified D-iLDH, the higher V_m and lower K_m values were now achieved with UQ_9H_2 (140±2 versus 64±10 (with RQ_9H_2) nmol (min × mg protein)$^{-1}$, (n=3); and 14.7±0.8 versus 32±10 μM (with RQ_9H_2), (n=3), respectively).

A similar proportion of total respiratory capacity by AOX activity was found in mitochondria from aerobic and microaerophilic cells (Table 1). Reconstitution of AOX activity with purified quinones in the presence of 0.015% triton X-100 caused a decrease of 60–75% in cyanide-resistant respiration. However, a RQ_9-H_2-dependent AOX activity of 11.5 ng atoms oxygen (min × mg protein)$^{-1}$ was detected, which was sensitive to nPG, a potent AOX inhibitor [33], and similar to that obtained with UQ_9H_2 (16 ng atoms oxygen (min × mg protein)$^{-1}$).

3.4. Reconstitution of electron transfer from D-lactate to cytochrome c

With purified D-iLDH and cytochrome bc_1 complex, reduction of cytochrome *c* in the presence of either RQ_9 or UQ_9 was achieved, which was sensitive to oxalate or antimycin, respectively (Fig. 5). The omission of any of the components (D-

Table 3
Kinetic constants for quinones of some dehydrogenases in *E. gracilis* and other organisms

Substrate	V_{max} (nmol (min × mg protein)$^{-1}$)/K_m (μM)		
	SDH	L-iLDH	D-iLDH
DBQ	22.4 (2)/13.8 (2)	116 (2)/13.8 (2)	142±14/34±4
UQ_1	252[a]/3[a]	106 (2)/14.3 (2)	130±12/32±2
UQ_2	34 (2)/15.2 (2)	114 (2)/13.5 (2)	132±9/32±4
UQ_9	26±3/16±2	92±9/14.3±1	121±12/45±6
RQ_9	n.d./>100	n.d./>100	151±26/15±3
RQ_{10}	n.m.	n.m.	125±16/19±1

Values shown are mean±S.D. of 3 different preparations except where is otherwise indicated between parentheses and represent V_{max}/K_m values. Values were determined at 30 °C in quinone-depleted mitochondria from aerobic *E. gracilis*. No activity was detected for SDH and L-iLDH using 100 μM of RQ_9. n.d., not detectable (<0.5 nmol (min × mg protein)$^{-1}$); n.m.; not measured.

[a] *A. suum* mitochondria from larval stage (L2) at room temperature [40].

Table 4
Kinetic constants for quinol of respiratory chain enzymes in *E. gracilis* and other organisms

Substrate	V_{max} (nmol (min × mg protein)$^{-1}$)/K_m (μM)		
	bc_1 complex	bc_1-*bypass*	AOX
DBQ	123 (2)/14.5 (2)	101±14/15±4	67 (2)/7.8 (2)
UQ_1	1920[a]/14.2[a]	n.m.	85±4/5±2
UQ_9	117±10/14±3	52 ±3/32±6	n.m.
RQ_9	54±3/32±5	16±9/64±5	n.m..
RQ_{10}	42±7/39±5	13±6/72±7	n.m.

Values shown are mean±S.D. of 3 different preparations except where is otherwise indicated between parentheses; and represent V_{max}/K_m values. Units for AOX V_m are nanogram atoms oxygen (min × mg protein)$^{-1}$. Values were determined at 30 °C in quinone-depleted mitochondria from aerobic *E. gracilis*. In mitochondria, cytochrome bc_1 complex and bc_1-*bypass* activities were calculated after addition of antimycin and myxothiazol as described in Materials and methods. n.m. not measured.

[a] *Sacharomyces cerevisiae*, values obtained at 36 °C [41].

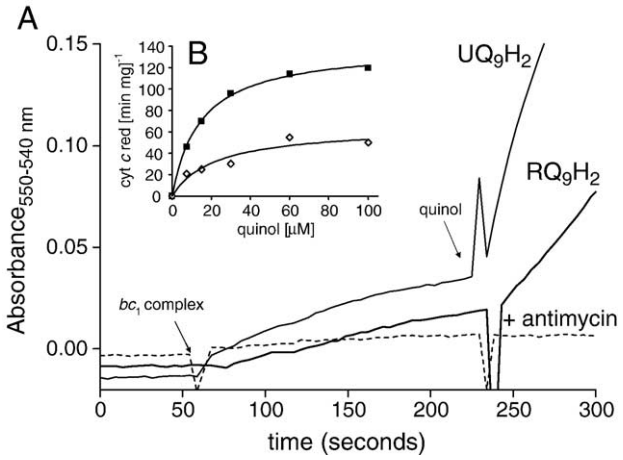


Fig. 4. Activity of purified *E. gracilis* cytochrome bc_1 complex with different quinones. (A) Representative spectrophotometric traces of quinone-dependent cytochrome *c* reduction with purified cytochrome bc_1 complex. Dotted line represents a control experiment in the presence of antimycin. Reaction was started with 50 μM reduced UQ_9 or reduced RQ_9. (B) Kinetic pattern of the reconstituted system with respect to UQ_9H_2 (■) or RQ_9H_2 (◇). An extinction coefficient of 19.1 mM^{-1} cm^{-1} was used to calculate the activities at each quinone concentration. Kinetic parameters were V_{max} 140±2.4 nmol cyt *c* red $(min \times mg)^{-1}$ and K_m 14.7±0.8 μM for reduced UQ_9 (■); and V_{max} 64.5±10 nmol cyt *c* red $(min \times mg)^{-1}$ and K_m 32±9.8 μM for reduced RQ_9 (◇).

iLDH, cytochrome bc_1 complex, D-lactate or quinone) brought up no cytochrome *c* reduction, except for the assay with D-lactate+D-iLDH, in which the activity corresponded to 10% of the activity measured in presence of RQ_9 (see legend to Fig. 5). Also, no activity was detected when L-lactate was added. The proportion of D-iLDH and cytochrome bc_1 complex used was based on the specific activities observed in *E. gracilis* mitochondria [7].

4. Discussion

4.1. Role of fumarate reductase

Many organisms have the ability to grow under aerobic and anaerobic conditions. Their mitochondrial electron transfer across the respiratory complexes diminishes under anaerobiosis and instead of oxygen; these organisms use other terminal acceptors, such as NO_3^-, NO_2^- [34] or fumarate [1]. The FR activity is coupled to low potential quinones such as menaquinone and RQ in these organisms [4]. *E. gracilis*, a facultative anaerobic and photosynthetic protist, can grow under low oxygen concentrations [26]. Moreover, *E. gracilis* possesses a significant content of RQ in its mitochondria, which suggests that the presence of RQ may be an adaptation for anaerobiosis [9].

Dark-grown *E. gracilis* showed a diminished cellular growth under microaerophilic conditions, which was associated with lower cytochrome content. Similar results were documented for *A. suum* mitochondria [1], in which diminution of all types of cytochrome appeared under anaerobiosis. The significant FR activity in aerobic *E. gracilis* mitochondria increased under microaerophilic conditions (Table 1) and it was accompanied by a slightly higher content of RQ_9 and a decrease in the UQ_9 content (Table 2). Similar variations in UQ and RQ content have been reported for *A. suum* mitochondria [1].

E. gracilis cells cultured under continuous light and with glucose as carbon source [9] have lower concentrations of UQ and RQ to those described in the present work with isolated *E. gracilis* mitochondria. In comparison to glutamate-malate medium, culturing dark-grown *E. gracilis* in glucose-medium limits its growth [32]. However, exposure of cells grown on glucose to microaerophilia also caused a similar variation in quinones content [9] to that described here for mitochondria isolated from cells grown on glutamate-malate.

In vitro, FR and SDH can generally catalyze both succinate oxidation and fumarate reduction, but at different rates [4]. The fumarate reduction determined in *E. gracilis* mitochondria was higher than the SDH activity under both aerobic and microaerophilic growth conditions, indicating that the formation of succinate was kinetically favored rather than its oxidation.

Due to their resemblance with quinone structure, HQNO and TTFA have been extensively used as inhibitors, although unspecific, of menaquinol-FR [29] and SDH [30], respectively. Thus, the insensitivity of FR to HQNO (a bacterial FR inhibitor) and partial sensitivity to the SDH-inhibitor TTFA indicated that FR in *Euglena* differs from bacterial menaquinol-FR. In larvae of *Strongyloides ratti*, a similar effectiveness to that found for *E. gracilis* mitochondria has been observed for TTFA when QFR and SDH activities are determined [35].

An increased content of intracellular succinate was found in agreement with the enhanced activity of FR observed under both high and low oxygen growth conditions. This result suggested that FR might function as an efficient anaplerotic reaction of the Krebs cycle to supply succinate under microaerophilic and also aerobic conditions; fumarate might proceed from malate dismutation.

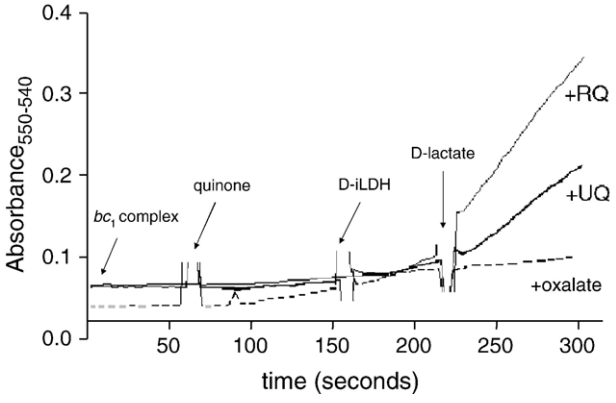


Fig. 5. Electron transfer reconstitution from D-lactate to cytochrome *c*. Experiments were made at 30 °C using 25 μg of purified cytochrome bc_1 complex, 50 μM RQ or UQ and 100 μg of purified D-iLDH . Reaction was started with 10 mM D-lactate. Dotted line represents a control experiment in the presence of oxalate. Non-enzymatic cytochrome *c* reduction (in the absence of cytochrome bc_1 complex) was no more than 5 nmol cyt *c* red min^{-1} mg^{-1} D-iLDH.

4.2. Microaerophilic metabolic changes

Pyruvate:$NADP^+$ oxidoreductase is a key enzyme for wax ester fermentation under anoxic conditions [36]. Although this enzyme is highly sensitive to oxygen, the effect of low oxygen concentrations on pyruvate:$NADP^+$ oxidoreductase is unknown. However, there was an enhanced secretion of L-lactate together with no variation in the intracellular content of D-lactate under microaerophilic conditions, which indicated no flux limitation from pyruvate to the lactate isomers. Thus, these observations suggested that pyruvate:$NADP^+$ oxidoreductase was not very active under microaerophilic conditions.

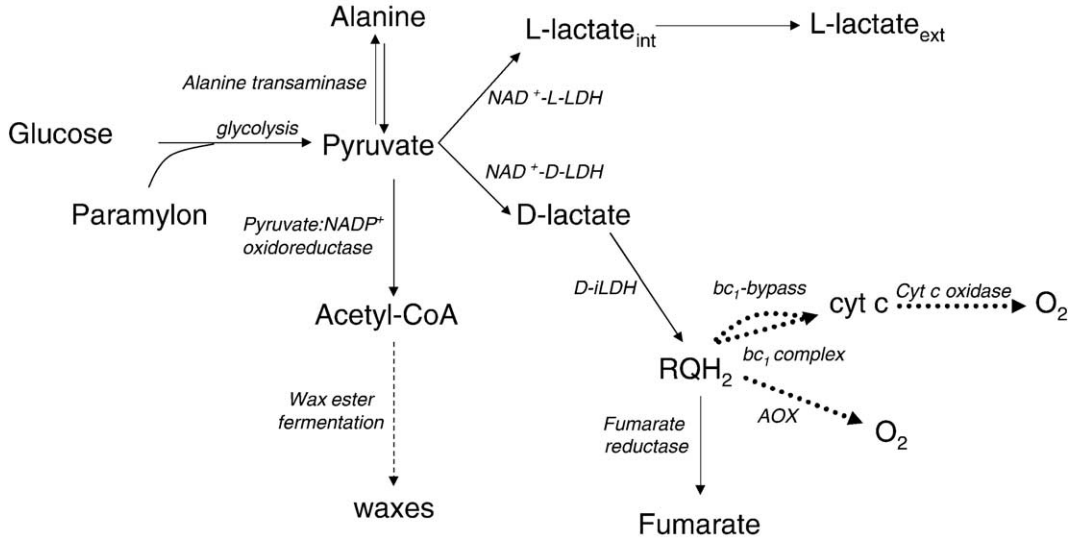
The AOX showed similar relative capacity under aerobic and microaerophilic conditions. Although AOX activity decreased in presence of detergent, preliminary data showed a similar oxidation with RQ_9H_2 or UQ_9H_2. In a previous report, a lower AOX affinity for oxygen was detected, in comparison to that of cytochrome *c* oxidase [7]. This last observation does not support a relevant role of AOX under microaerophilic conditions. In contrast, AOX seems relevant under oxidative stress conditions [7].

D-iLDH was the predominant dehydrogenase under microaerophilic conditions. The reasons for this pattern are unknown. Why does *E. gracilis* exposed to microerophilia develop a metabolism based on D-lactate? Perhaps, this behavior is related to an increased glycolytic flux at low O_2 concentrations (Pasteur effect) that leads to a higher synthesis of D-lactate under microaerophilia. Formation of D-lactate from methylglyoxal by the glyoxalase system is present in some bacteria and eukaryotic cells [37,38]. Methylglyoxal, formed from dihydroacetonephosphate by methylglyoxal synthase, increases during stressful episodes [37]. Although a decrease in glutathione content has been described in *Euglena* during cold exposure [7], the association of this compound with methylglyoxal and the hydrolysis of the resulting lactoyl-glutathione by the glyoxalase system to form D-lactate and glutathione have not been explored in *E. gracilis* cells. An increased flux through this pathway under low oxygen conditions might result in a more relevant role for D-lactate as an essential metabolite.

4.3. Role of rhodoquinone

Analysis of the kinetic parameters revealed that the typical respiratory components cytochrome bc_1 complex and SDH as well as the alternative components *bc_1-bypass* and L-iLDH have preference for the high potential UQ (Tables 3 and 4). For example, cytochrome bc_1 complex was 5 times more efficient with UQ (Table 4). In contrast D-iLDH showed preference for the low potential RQ. Thus, both mitochondrial and purified D-iLDH exhibited 3.4–3.7 higher catalytic efficiency values (V_{max}/K_m) with RQ_9 than with UQ_9. Moreover, the reduction quinone analysis (*cf.* Fig. 2B) indicated that among the dehydrogenases studied only D-iLDH transfers reducing equivalents to the mitochondrial electron transfer chain through the RQ pool.

In *A. suum*, complex I is able to use UQ as well as RQ under aerobic and anaerobic conditions, respectively [39]. These authors [39] demonstrated that the two quinones bind differently to complex I by analyzing the inhibition mechanism by quinazolines, and the pH and structure–activity profile with both quinones. The binding of quinazoline at the quinone-binding site blocks the RQ binding. At alkaline pH, the inhibition profile of quinazoline becomes non-competitive for UQ, whereas for RQ remains competitive [39]. No studies about the RQ and UQ binding to D-iLDH have been made since no preferential use of quinones by dehydrogenases had been previously reported. The same authors also reported an oxidation of NADH in bovine sub-mitochondrial particles mediated by the artificial electron acceptor RQ_2 [39], with a significant substrate inhibition at high substrate concentrations. In *E. gracilis*, the reduction of RQ by D-iLDH under aerobiosis did not show significant substrate inhibition up to 100 μM.



Scheme 1. Proposed model of electron transfer involving RQ in microaerophilic heterotophic *Euglena*.

Scheme 1 summarizes the results reported in the present work. D-iLDH preferentially reduces RQ, which transfers electrons to FR and also, with a lower efficiency, to cytochrome bc_1 complex, bc_1-*bypass*, and AOX. Under microaerophilic conditions, the rate of fumarate reduction predominates over that of quinone pool oxidation by the aerobic chain. Rhodoquinol is presumably the electron donor for fumarate reduction, as reported for other organisms [4]. However, D-lactate is the main electron source for rhodoquinone reduction in *E. gracilis*, in contrast to *A. suum* [39], which use NADH as the electron donor.

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References

[1] S. Takamiya, K. Kita, H. Wang, P.P. Weinstein, A. Hiraishi, H. Oya, T. Aoki, Developmental changes in the respiratory chain of *Ascaris* mitochondria, Biochim. Biophys. Acta 1141 (1993) 65–74.

[2] J.J. Van Hellemond, M. Luijten, F.M. Flesh, C.P. Gaasenbeek, A.G. Tielens, Rhodoquinone is synthesized de novo by *Fasciola hepatica*, Mol. Biochem. Parasitol. 82 (1996) 217–226.

[3] H. Miyadera, A. Hiraishi, H. Miyoshu, K. Sakamoto, R. Mineki, K. Murayama, K.V. Nagashima, K. Mtsuura, S. Kojima, K. Kita, Complex II from phototrophic purple bacterium *Rhodoferax fermentans* displays rhodoquinol-fumarate reductase, Parasitology 115 (1997) 177–182.

[4] C. Hagërhäll, Succinate:quinone oxidoreductases, variations on a conserved theme, Biochim. Biophys. Acta 1320 (1997) 107–141.

[5] R. Moreno-Sánchez, R. Covián, R. Jasso-Chávez, S. Rodríguez-Enríquez, F. Pacheco-Moisés, M.E. Torres-Márquez, Oxidative phosphorylation supported by an alternative respiratory pathway in mitochondria from *Euglena*, Biochim. Biophys. Acta 1457 (2000) 200–210.

[6] R. Jasso-Chávez, M.E. Torres-Márquez, R. Moreno-Sánchez, The membrane-bound L- and D-lactate dehydrogenase activities in mitochondria from *Euglena gracilis*, Arch. Biochem. Biophys. 390 (2001) 295–303.

[7] N.A. Castro-Guerrero, K. Krab, R. Moreno-Sánchez, The alternative respiratory pathway of *Euglena* mitochondria, J. Bioenerg. Biomembr. 36 (2004) 459–469.

[8] D.R. Threlfall, Incorporation of L-[Me-$^2H_3$] methionine into isoprenoid quinones and related compounds by *Euglena gracilis*, Biochim. Biophys. Acta 280 (1972) 472–480.

[9] M. Hoffmeister, A. van der Klei, C. Rotte, K.W.A. van Grinsven, J.J. van Hellemond, K. Henze, A.G.M. Tielens, W. Martin, *Euglena gracilis* rhodoquinone: ubiquinone ratio and mitochondrial proteoma differ under aerobic and anaerobic conditions, J. Biol. Chem. 279 (2004) 22422–22429.

[10] C.L. Greenblatt, J.A. Schiff, A pheophytin—Like pigment in dark-adapted *Euglena gracilis*, J. Protozool. 6 (1959) 23–28.

[11] A. Uribe, R. Moreno-Sánchez, Energy-dependent reactions supported by several substrates in coupled *Euglena gracilis* mitochondria, Plant Sci. 86 (1992) 21–32.

[12] K.A. Gray, M. Grooms, H. Myllykallio, C. Moomaw, G. Slaughter, F. Daldal, *Rhodobacter capsulatus* contains a novel *cb*-type cytochrome *c* oxidase without a CuA center, Biochemistry 33 (1994) 3120–3127.

[13] M. Degli Esposti, G. Lenaz, The kinetic mechanism of ubiquinol: cytochrome *c* reductase at steady state, Arch. Biochem. Biophys. 289 (1991) 303–312.

[14] J.W. Priest, J.W. Hajduk, Cytochrome *c* reductase purified from *Crithidia fasciculate* contains an atypical cytochrome c_1, J. Biol. Chem. 267 (1992) 20188–20195.

[15] H. Ding, D.E. Robertson, F. Daldal, P.L. Dutton, Cytochrome bc_1 complex [2Fe–2S] cluster and its interaction with ubiquinone and ubihydroquinone at the Qo site: a double-occupancy Qo site model, Biochemistry 31 (1992) 3144–3158.

[16] A.M. Wagner, M.J. Wagner, Measurements of in vivo ubiquinone reduction levels in plant cells, Plant Physiol. 108 (1995) 277–283.

[17] J.M. Armstrong, The molar extinction coefficient of 2, 6-dichlorophenol indophenol, Biochim. Biophys. Acta 86 (1964) 194–197.

[18] R.L. Lester, Y. Hatefi, C. Widmer, F.L. Crane, Studies on the electron transport system. XX. Chemical and physical properties of the coenzyme Q family of compounds, Biochim. Biophys. Acta 33 (1959) 169–185.

[19] J. Glover, D.R. Threfall, A new quinone (rhodoquinone) related to ubiquinone in the photosynthetic bacterium *Rhodospirillum rubrum*, Biochem. J. 85 (1962) 14p.

[20] R. Jasso-Chávez, I. García-Cano, A. Marín Hernández, D. Mendoza-Cózatl, J.L. Rendón, R. Moreno-Sánchez, The bacterial-like lactate shuttle components from heterotrophic *Euglena gracilis*, Biochim. Biophys. Acta 1709 (2005) 181–190.

[21] I. Kucera, M. Kunak, Steady-state kinetics analysis of substrate pair cycling between two enzymes: application to a mediated electron transport between the cytoplasmic membrane and the periplasmic nitrite reductase of *Paracoccus denitrificans*, Biophys. Chem. 104 (2003) 617–622.

[22] B.L. Trumpower, C.A. Edwards, Purification of a reconstitutively active iron–sulfur protein (oxidation factor) from succinate-cytochrome c reductase complex of bovine heart mitochondria, J. Biol. Chem. 254 (1979) 8697–8706.

[23] R. Covián, J.P. Pardo, R. Moreno-Sánchez, Tight binding of inhibitors to bovine bc1 complex is independent of the Rieske protein redox state, J. Biol. Chem. 277 (2002) 48449–48455.

[24] H.U. Bergmeyer, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. 3–9, Weinheim Verlag Chemie, Germany, 1983.

[25] W.S. Thayer, E. Rubin, Effects of chronic ethanol intoxication on oxidative phosphorylation in rat liver submitochondrial particles, J. Biol. Chem. 254 (1979) 7717–7723.

[26] J.B. Lackey, Ecology of *Euglena*, in: D.E. Buetow (Ed.), The Biology of *Euglena*, vol. 1, Academic Press, New York, 1968, p. 35.

[27] M.R. Weickowski, L. Wojtczak, Fatty acid-induced uncoupling of oxidative phosphorylation is partly due to opening of the mitochondrial permeability transition pore, FEBS Lett. 423 (1998) 339–342.

[28] A. Baracca, G. Sgarb, G. Solaini, G. Lenaz, Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F0 during ATP synthesis, Biochim. Biophys. Acta 1606 (2003) 137–146.

[29] E. Maklashina, G. Cecchini, Comparison of catalytic activity and inhibitors of quinone reactions of succinate dehydrogenase (succinate-ubiquinone oxidoreductase) and fumarate reductase (menaquinol-fumarate oxidoreductase) from *Escherichia coli*, Arch. Biochem. Byophys. 369 (1999) 223–232.

[30] T. Schewe, C. Hiebsch, Action of respiratory inhibitors on the electron transport system of *Escherichia coli*, Acta Biol. Med. Ger. 36 (1977) 961–966.

[31] A.B. Kotlyar, A.D. Vinogradov, Interaction of the membrane-bound succinate dehydrogenase with substrate and competitive inhibitors, Biochim. Biophys. Acta 784 (1984) 24–34.

[32] R. Jasso-Chávez, R. Moreno-Sánchez, Cytosol–mitochondria transfer of reducing equivalents by a lactate shuttle in heterotrophic *Euglena*, Eur. J. Biochem. 270 (2003) 4942–4951.

[33] J.N. Siedow, M.E. Girvin, Alternative respiratory pathway: its role in seed respiration and its inhibition by propyl gallate, Plant Physiol. 65 (1980) 669–674.
[34] A.G.M. Tielens, C. Rotte, J.J. van Hellemond, W. Martin, Mitochondria as we don't know them, Trends Biochem. Sci. 27 (2002) 564–572.
[35] A. Armson, W.B. Grubb, A.H. Mendis, The effect of electron transport (ET) inhibitors and thiabenzadole on the fumarate reductase (FR) and succinate dehydrogenase (SDH) of *Strongyloides ratti* infective (L3) larvae, Int. J. Parasitol. 25 (1995) 261–263.
[36] H. Inui, K. Miyatake, Y. Nakano, S. Kitaoka, The physiological role of oxygen-sensitive pyruvate dehydrogenase in mitochondrial fatty acid synthesis in *Euglena gracilis*, Arch. Biochem. Biophys. 237 (1985) 423–429.
[37] I.R. Booth, G.P. Ferguson, S. Miller, C. Li, B. Gunasekera, S. Kinghorn, Bacterial production of methylglyoxal. A survival strategy or death by misadventure? Biochem. Soc. Trans. 31 (2003) 1406–1408.
[38] A. Ponces Freire, A. Ferreira, R. Gomes, C. Cordero, Anti-glycation defenses in yeast, Biochem. Soc. Trans. 31 (2003) 1409–1412.
[39] T. Yamashita, T. Ino, H. Miyoshi, K. Sakamoto, A. Osanai, E. Nakamaru-Ogiso, K. Kita, Rhodoquinone reaction site of mitochondrial complex I, in parasitic helminth, *Ascaris suum*, Biochim. Biophys. Acta 1608 (2004) 97–103.
[40] F. Saruta, T. Kuramochi, K. Nakamura, S. Takamiya, Y. Yu, T. Aoki, K. Sekimizu, S. Kojima, K. Kita, Stage-specific isoforms of complex II (succinate-ubiquinone oxidoreductase) in mitochondria from the parasitic nematode *Ascaris suum*, J. Biol. Chem. 270 (1995) 928–932.
[41] M. Cavazzoni, J. Svobodova, A. De Santis, R. Fato, G. Lenaz, Steady-state kinetics of ubiquinol-cytochrome c reductase in *Sacharomyces cerevisiae* mitochondria: effects of fluidity changes obtained by different growth temperatures, Arch. Biochem. Biophys. 303 (1993) 246–254.

DATOS NO MOSTRADOS EN EL ARTÍCULO *Biochimica Biophysica Acta- Bioenergetics* Vol: 1710 (2005) pág. 113-121.

(**1**) Las células de *E. gracilis* no se duplicaron cuando la concentración de O_2 alcanzó valores menores a 63 µM (Pág. 115).

La concentración de oxígeno más baja (52 µM) fue obtenida al burbujear suavemente N_2 por 30 min antes de esterilizarlo y sonicarlo por 20 min. En estos medios de cultivo no se observó crecimiento celular. Como control, un medio tratado de la misma forma fue aireado por agitación, sin comprometer su esterilidad. Después de 24 h se midió un aumento en la concentración de oxígeno (108 µM, n=1) y en este medio sí se registró crecimiento celular.

(**2**) En mitocondrias provenientes de células microaerofílicas no se detectó potencial eléctrico transmembranal con los diferentes sustratos utilizados mediante la técnica de safranina O ó rodamina 123 (Pág. 115).

Estos resultados fueron obtenidos con los tres diferentes sustratos utilizados: succinato, L- y D-lactato. La concentración de proteína utilizada osciló entre 0.450- 1 mg de proteína mitocondrial.

En la Fig. 8 se ejemplifica la determinación del potencial de membrana con 0.25 µM de rodamina 123 en 0.6 mg de mitocondrias control y provenientes de células de cultivos microaerofílicos a 30°C en 2 mL de amortiguador KME utilizando 503 y 527 nm como longitudes de onda de excitación y emisión respectivamente.

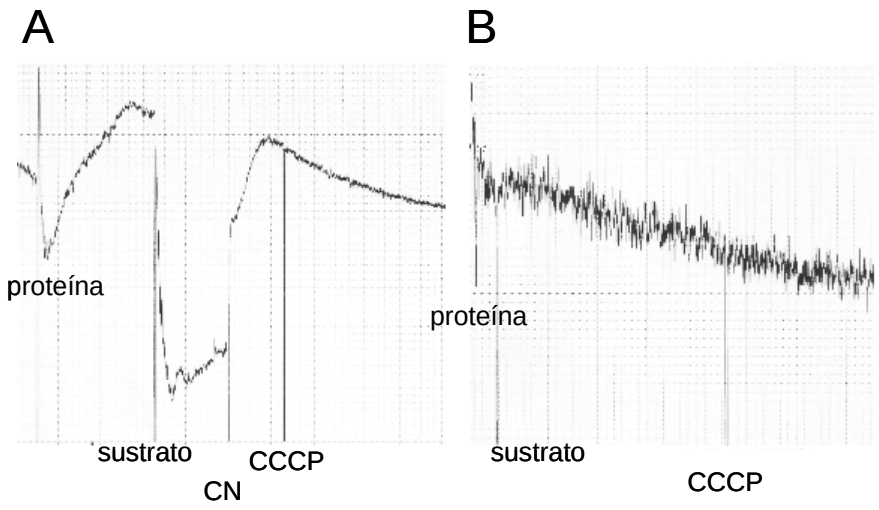

Figura 8. - Determinación del potencial de membrana. A) Mitocondrias provenientes de cultivos control; B) Mitocondrias provenientes de cultivos microaerofílicos.

(**3**) Con D-lactato el cianuro ocasionó un aumento de 84 % en la poza de la rodoquinona. (Pág. 116).

A diferencia del succinato y del L-lactato, la oxidación del D-lactato sí promovió la reducción de la rodoquinona en mitocondrias de *E. gracilis*. Además, fue notorio encontrar que el estado de reducción de la rodoquinona es muy alto (60%). Este porcentaje aumentó aproximadamente a 84 % en presencia de cianuro.

Aunque este último porcentaje no representa el máximo de reducción de la poza, sí sugiere que parte de la oxidación de este sustrato a través de la rodoquinona tiene como destino final el complejo IV, el cual es sensible a cianuro.

(**4**) La inhibición por la antimicina fue similar para la UQ o la RQ; la actividad remanente se abatió con el mixotiazol (Pág. 116).

Tabla 4. Inhibición de la actividad de citocromo *c* reductasa.

	cit *c* red (min mg)$^{-1}$	**Complejo *bc*$_1$ + 0.5 µM antimicina**	**+ 0.5 µM antimicina + 10 µM mixotiazol**
UQ-9	**176 ± 36**	**117 ± 10 (66 %)**	**0 ± 0**
RQ-9	**72 ± 7**	**54 ± 3 (69 %)**	**0 ± 0**

Valores correspondientes al promedio ± SD de 3 experimentos, medidos a 30 °C y utilizando las quinonas reducidas.

-EXPOSICION A CADMIO

RESUMEN DEL TRABAJO

Anteriormente, las evidencias generadas en el modelo de baja temperatura, tratamiento con cianuro y exposición a H_2O_2 mostraron que la actividad de la AOX puede ser relevante en condiciones de estrés. La última condición usada es el modelo de exposición a cadmio que refleja una condición a la que ambientalmente este protista puede estar expuesto (Hargreaves JW, Lloyd EJH, Witton BA (1975) Chemistry and vegetation of highly acidic streams, Freshw Biol 5: 563-576).

Inicialmente, se definió la concentración de cadmio para trabajar. Al determinarse una IC_{50} de 30 µM, se decidió manejar como condición de estrés la concentración de 50 µM, en la que se observó un menor crecimiento y donde los cambios celulares podrían ser más evidentes. Al evaluar el estrés generado por esta concentración de cadmio se encontró un aumento en la lipoperoxidación, un aumento en la oxidación de la poza del glutatión y un aumento en la poza de las quinonas.

Además, encontramos que por efecto del cadmio, sucedía un aumento importante en la concentración del hierro y del cobre, principales precursores de la reacción de Fenton y por lo tanto posibles generadores de EROs.

Como contraparte, encontramos un aumento de la respuesta antioxidante presente en *E. gracilis* como cabría esperar en una condición de estrés.

De forma interesante, la respiración celular se mantuvo inalterada, aunque la resistencia al cianuro fue mayor.

En la mitocondria, evaluamos una disminución drástica en la actividad de la enzima aconitasa, la cual es utilizada como un monitor de estrés oxidativo debido a la alta sensibilidad que tiene hacia metabolitos oxidativos como el óxido nítrico. En la cadena respiratoria se encontraron disminuidas las actividades y los contenidos de los complejos III y IV.

En forma contraria, la AOX aumentó su actividad y su concentración. Estos resultados apoyaron la hipótesis de la función de la AOX en modular la generación de EROs, permitiendo que la cadena respiratoria siga funcionando y exista un aporte de los precursores biosintéticos, los cuales fueron evaluados y se evidenció que compuestos fosfatados como el ATP y el pirofosfato (de alta energía) e intermediarios de la glucólisis,

no fueron alterados. Es importante mencionar que sí hubo una disminución en las triosas fosfato posiblemente por la inhibición de la aldolasa, la cual, en la cepa heterotrófica de *E. gracilis* es principalmente de clase II. Este tipo de aldolasas son metaloenzimas y el cadmio podría ejercer un efecto inhibitorio directo. También existen algunos antecedentes sobre la inhibición de la síntesis de la GAPDH en el alga *N. oculata* (Kim YK, Yoo WI, Lee SH, Lee MY (2005) Proteomic analysis of cadmium-induced protein profile alterations from marine alga *Nannochloropsis oculata*, Ecotoxicology 14: 589-596.) o de su actividad en mamíferos (Casalino E, Calzaretti G, Sblano C Landriscina C (2000) Cadmium-dependent enzyme activity alteration is not imputable to lipid peroxidation, Arch. Biochem. Biophys. 383: 288-295), aunque en ambos casos el efecto es menor en comparación a otras deshidrogenasas como la SDH.

ARTÍCULO en preparación

Enhanced alternative oxidase and antioxidant enzymes under Cd^{2+} stress in *Euglena*

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Running Title: Cellular response to Cd^{2+} stress in *Euglena gracilis*

Key Words: oxidative stress; heavy metal homeostasis; alternative oxidase; Cu; Fe.

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Abstract

The effect of Cd^{2+} on the energy metabolism, oxidative stress and the heavy metal homeostasis of heterotrophic *Euglena gracilis* was studied. $CdCl_2$ (50 μM) induced: (i) diminution in cell growth, concentrations of ADP, fructose-1,6-bisphosphate, and trioses phosphate, and contents of zinc, magnesium and calcium; (ii) increase in the cell contents of manganese, iron, cobalt, copper, potassium, malondialdehyde and oxidized glutathione; and in the activities of ascorbate peroxidase, glutathione peroxidase and glutathione reductase. However, Cd^{2+} stress did not modify the cellular respiratory rate or the intracellular levels of ATP, PPi, glucose-6-phosphate, fructose-6-phosphate and AMP. In isolated mitochondria, the Cd^{2+} effect consisted in a marked decrease in the activities of aconitase (a marker of oxidative stress) and respiratory complexes III and IV, and in the contents of iron and copper. The mitochondrial response to Cd^{2+} stress involved an enhanced capacity of the alternative oxidase (AOX) in both intact cells and isolated mitochondria; in fact, AOX activity accounted for 69-91 % of total respiration. Western blotting also revealed an increased AOX content in mitochondria from Cd^{2+}-exposed cells. Moreover, AOX was more resistant to Cd^{2+} inhibition than cytochrome c oxidase in mitochondria from control and Cd^{2+}-exposed cells. Therefore, the data of the present work indicated that, in addition to promoting a significant oxidative stress and hence inhibition of cell growth, Cd^{2+} stress also induced a strong perturbation of the cellular metal homeostasis. However, the response developed by the Cd^{2+}-exposed cells, consisting in an increased anti-oxidant enzyme activity that included the AOX, was still able to maintain unaltered the energy status.

1. Introduction

Heavy metals may induce oxidative stress through different mechanisms, depending on their physical and chemical properties [1-3]: by directly inducing reactive oxygen species (ROS) production (Fenton reaction), by blocking functional groups of proteins and glutathione, or by displacing essential metals like zinc or selenium from proteins [4] and zinc from zinc-finger motifs of transcription factors [5, 6]. Thus, the growth of some organisms in heavy-metal polluted environments depends on their ability to synthesize different heavy-metal chelating molecules and activate efficient antioxidant mechanisms.

Cadmium enters the environment from industrial waste and is one of the most toxic heavy metals for living beings. The main route of toxicity for this metal is through the depletion of glutathione and the binding to other thiol groups in proteins [1]; it also may induce the production of lipid peroxides [1, 4]. In mammals, Cd^{2+} is taken up by mitochondria thus inhibiting cellular respiration and oxidative phosphorylation through ROS-mediated mechanisms [4, 7]. Other Cd^{2+}-sensible cellular processes are photosynthesis [8-10], membrane lipid composition [11, 12] and the uptake of external nutrients [13].

Euglena gracilis is a free-living protist that tolerates high concentration of different heavy-metals [14-16]. An increased ROS production by Cd^{2+} exposure in dark-grown and photosynthetic strains of *E. gracilis* has been documented [17, 18]. Interestingly, most of the accumulated Cd^{2+} is localized in chloroplasts of photosynthetic cells [16, 19] and mitochondria of dark-grown cells [20], which imply a direct toxic effect over these organelles and their energy metabolism: photo- and oxidative phosphorylation. Mitochondrial Ca^{2+} uptake and release is also directly affected by Cd^{2+} in *E. gracilis* [21].

In most organisms, catalase (CAT) and superoxide dismutase (SOD) participate in the detoxification of H_2O_2 and superoxide, thus avoiding the formation of $OH^{\bullet}$ radicals. However, in *E. gracilis*, CAT [22] as well as mitochondrial and cytosolic SOD [23] is absent. These observations suggest a fragile antioxidant system or the presence of other mechanism by which *E. gracilis* maintains its redox balance. On this regard, a chloroplastic SOD [23], two cytosolic glutathione peroxidases (GPx) [24] and a complete ascorbate-glutathion cycle have been characterized as part of the antioxidant mechanism present in *E. gracilis* [25, 26]. Furthermore, a trypanothione reductase has also been described, which may be involved in the antioxidant response [27]. Information about other enzymes involved in trypanothione metabolism in this protist is unknown.

E. gracilis has a complex respiratory chain constituted by different alternative and classical components, in which the alternative oxidase (AOX) seems essential when cells are subjected to oxidative stress, induced either by cold or respiratory inhibitors [28]. It is well documented for many organisms [17, 18, 29] that exposure to Cd^{2+} increases the ROS production and then AOX might also be involved in the *E. gracilis* response. To gain insight on the defense mechanisms of *E. gracilis* under Cd^{2+} stress a variety of cellular and mitochondrial processes including the AOX and the anti-oxidant enzymes and metabolites present in this protist were evaluated.

2. Materials and methods

Chemicals

L- lactate, N, N, N', N'-tetramethyl-p-phenylendiamine (TMPD), 1, 6-dichlorophenol indophenol (DCPIP), 5, 5' -dithio-bis(2-nitrobenzoic acid) (DTNB), NADH, glutathione reductase, GSH (reduced glutathione), ascorbic acid, citrate and *tert*-butyl hydroperoxide (TBHP) were purchased from Sigma Chem. Co. Sodium

cyanide was from J.T. Baker, whereas n-propyl gallate, GSSG (oxidized glutathione) and horse heart cytochrome *c* were from ICN Biomedicals Inc. NADPH, hexokinase, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase and lactate dehydrogenase were from Roche Co. Purified recombinant pyruvate phosphate dikinase from *E. histolytica* was kindly provided by Dr. Emma Saavedra (Instituto Nacional de Cardiología de México).

Culture conditions

Cells of *E. gracilis* Z were grown in the dark in the Hutner´s acidic organotrophic medium with glutamate + malate as carbon source [30] at 25 ± 2 ºC under orbital shaking (125 rpm). $CdCl_2$ was added to the culture medium before the cellular inoculum.

Mitochondria were isolated as previously described [28] from cells reaching the stationary phase (96 h of culture).

Determination of metabolites

The intracellular content of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, ATP, and trioses phosphate (glyceraldehyde-3-phosphate + dihydroacetonephosphate) were estimated by standard enzymatic assays [31] in cell extracts from suspensions of 1×10^8 cells mixed with 3 % (v/v) perchloric acid and further centrifuged and neutralized with 3 M KOH/ 0.1 M Tris.

Pyrophosphate was determined at 37 °C in 50 mM imidazole plus 10 mM each of acetate, MES and Tris, pH 6, 0.15 mM NADH, 5 mM $MgCl_2$, 0.5 mM AMP, 1 mM PEP, 1.5 U/ml LDH and 0.15 U/ml *Entamoeba histolytica* pyruvate phosphate dikinase.

Metals were determined in acid-digested fractions (from 3 x 10^7 cells harvested after 4 days of culture) by atomic absorption spectrophotometry in a SpectrAA 640 (Varian Australia Pty Ltd.) spectrophotometer as described previously [14, 16].

Malondialdehyde (MDA) was determined by measuring the concentration of thiobarbituric acid-reacting substances (TBARS) as described previously [32].

The content of reduced glutathione (GSH) was determined by reverse phase-HPLC (RP-HPLC) coupled to post-column derivatization with DTNB (Ellman´s reagent) as described previously [16] in fresh acid extractions from 3 x 10^7 cells. Total glutathione (GSH+GSSG) was determined by reducing the samples with 1 mM DTT before acid extraction in the presence of 0.05 % (v/v) Triton X-100.

Enzyme activities

Cellular and mitochondrial respiration in stressed and non-stressed cells was determined at 25 °C by means of a Clark-type oxygen electrode, in air-saturated KME (120 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.25) buffer.

Respiration, pyridine nucleotide-independent L-lactate dehydrogenase (L-iLDH) and cytochrome *c* reductase activities in intact fresh mitochondria were determined at 25 °C as previously described [28, 33]. For the estimation of the AOX capacity, 1 mM cyanide and 3 mM n-propyl gallate (nPG) were added in respiration experiments. TMPD (cytochrome c) oxidase activity was evaluated by determining the rate of O_2 uptake after adding 5 mM ascorbate plus 2.5 mM TMPD, which was fully inhibited by 10 mM azide or 1 mM cyanide [28]. For the Cd^{2+}-sensitivity assays, freshly prepared mitochondria were incubated in the absence of EGTA and with 120 mM KCl, 20 mM MOPS, pH 7.25. Because mitochondria suspended in this buffer rapidly deteriorated, the assays were carried out for short times. The free Cd^{2+} concentration was calculated by using the software CHELATOR [34], and taking into account the EGTA added with

the mitochondrial sample. The oxygen solubility was determined to be 420 ng atoms/ml (210 µM) at 25 °C and 2240 m altitude.

Aconitase activity was measured as the formation of *cis*-aconitate (extinction coefficient ε= 3.6 mM^{-1} cm^{-1}) from citrate at 240 nm [35]. The reaction mixture contained 10 mM citrate and 0.2 mM $NADP^+$. The reaction was started by adding the mitochondrial protein; the range of protein concentration under which the rate was linear was from 50 to 150 µg. Control reactions in the absence of citrate or protein were made and they indicated no unspecific activity. Mn^{2+} was not added in the reaction mixture since it showed an inhibitory effect in our model (0.6 mM $MnCl_2$ inhibited 50 % aconitase activity in *E. gracilis* mitochondrial preparations).

GPx activity was determined in cell extracts by measuring the disappearance of 100 µM NADPH in a reaction coupled to the reduction of 1 mM *ter*-butyl hydroperoxide (TBHP) by glutathione reductase (0.4 U), with 5 mM GSH as electron donor [36]. The rate was linear for the range of protein concentration from 25 to 150 µg. Use of H_2O_2 instead of TBHP also revealed GPx activity, although the *Km* value was higher. The absence of either substrate did not yield activity.

APx activity was determined according to the method of Nakano and Asada [37] in cell extracts prepared in the presence of 2 mM ascorbate. The reaction was followed by disappearance of 0.5 mM freshly prepared ascorbate at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) starting the reaction with 2 mM H_2O_2 [38]. The range of protein concentration for rate linearity was from 25 to 100 µg. No activity was registered in the absence of ascorbate. A low activity (< 5% of total) was obtained before the addition of H_2O_2; the APx activity was corrected for this basal activity.

GR activity was determined according to Shigeoka *et al.* [25] in cell extracts by measuring the oxidation of 0.2 mM NADPH at 340 nm in the presence of 1 mM GSSG

at 25 °C. In reaction without added GSSG no consumption of NADPH occurred. Rate linearity was achieved from 25 to 150 μg protein.

Triose phosphate isomerase (TPI) in cell extracts (0.1-0.7 μg protein) was determined in the reverse reaction in 20 mM Tris pH 8.0, 0.15 mM NADH, and 1.7-3.4 U glycerol-3-phosphate dehydrogenase. The reaction was started by adding 1.3 mM glyceraldehyde-3-phosphate.

Western blotting

Immunodetection was made by using an antibody raised against AOX from *Chlamydomonas reinhardtii*. For blotting, the mitochondrial extracts (50 μg protein) were first subjected to SDS-PAGE and then the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated overnight with the anti-AOX antibody at 4°C. Then, the membrane was incubated with a second antibody conjugated with horseradish peroxidase for 1 h at room temperature and. The protein was detected by using the ECL detection system kit (Amersham Biosciences). As control, mitochondrial adenine nucleotide translocator (ANT) was detected in the same membrane with a polyclonal human antibody (Santa Cruz). The anti-ANT antibody was used after removing the antibodies of the initial reaction from the membrane using a stripping buffer composed by 2% SDS, 62.5 mM Tris and 100 mM β-mercaptoethanol at pH 6.7 and further incubating at 50 °C for 20 min.

3. Results

Cellular growth of heterotrophic *E. gracilis* under Cd^{2+} stress has been previously documented, but in culture media containing lactate as carbon source [19]. Culture with glutamate + malate as carbon source and in the presence of different $CdCl_2$ concentrations progressively altered cell growth. Inhibitory concentration for 50

% decrease in cellular density after 4 days (IC_{50}) in media with glutamate + malate was 30 ± 3 µM (n=3) $CdCl_2$. In Cd^{2+}-exposed cells, the rate of cellular respiration was identical to that attained by control cells. However, the rate of cyanide-resistant respiration accounted for 18 % and 69 % of total respiration for control and Cd^{2+}-stressed cells, respectively (Table 1) , suggesting an enhanced activity of AOX under Cd^{2+} stress.

Surprisingly, the intracellular concentrations of the high-energy related metabolites ATP, PPi, G6P and F6P, and AMP were not modified by Cd^{2+} stress (Table 1). In fact, such a strong homeostatic control consisted in an *increase* in the metabolite content as the intracellular water volume in Cd^{2+} stress *E. gracilis* cells is approximately 3-fold higher than in control cells [20]. In turn, the concentrations of ADP, F-1,6-BP and trioses phosphate diminished in Cd^{2+}-exposed cells..

Heavy metal homeostasis

Cadmium induces alterations in the composition of trace elements in plants [13]. Perturbation of the Cu and Fe homeostasis may promote oxidative stress through the activation of the Fenton reaction. In order to evaluate a possible impairment in the heavy metal homeostasis in *E. gracilis* induced by Cd^{2+}, the intracellular content of several trace nutrient heavy metals was determined. It should be noted that in *Euglena* cultures, the medium is constituted by a variety of nutrients such as Zn, Mn, Fe and Cu, which are present at relatively high concentrations (Table 2). Due to the very high affinity of functional groups (carboxylate, sulhydrilic, imidazole) in biomolecules for heavy metals [39], their internal concentrations are difficult to determine although they are expected to be in the micromolar and nanomolar range. Therefore, despite knowing the intracellular water volumes, the data in Table 2 were only expressed in terms of content. Nickel is not added to the culture medium but it is a contaminant from other

components; although this contaminant was significant, the cell content of this metal remained constant in both types of cells (Table 2).

Except for Zn, all other heavy metals determined (Mn, Fe, Co, Cu) increased when cells were exposed to Cd^{2+}, being Cu and Fe, inorganic catalysts for ROS production, the most affected (Table 2). The mitochondrial content of Cu and Fe increased, respectively, from 0.5 ± 0.1 (n=3) and 2.8 ± 2 (n=7) nmol (mg protein)$^{-1}$ in control mitochondria to 3 ± 0.2 (n=7) and 10.5 ± 2 (n=9) nmol (mg protein)$^{-1}$ in mitochondria from Cd^{2+}-cultured cells.

Variation in the intracellular zinc content correlated with a lowering in the Zn^{2+} concentration in the culture medium from 468 ± 38 µM (concentration at the beginning of the growth curve; n=3) to 44.6 ± 17 µM (n=4) after 96 h in control cells, and to 178 ± 23 µM (n=5) in Cd^{2+}-exposed cells. In mitochondria isolated from control and Cd^{2+}-exposed cells, the Zn^{2+} content was 14 ± 7 (n=6) and 9.3 ± 5 nmol (mg protein)$^{-1}$ (n=8), respectively.

The cellular content of Mg^{2+} and Ca^{2+} diminished, and that of K^{+} increased under Cd^{2+} stress (Table 2). In turn, the contents of Mg^{2+}, Ca^{2+} and K^{+} in control mitochondria were 123 ± 43 (n=8), 19 ± 14 (n=6) and 21.5 ± 12 (n=6) nmol (mg protein)$^{-1}$, respectively, which diminished by 85, 64 and 66 % in mitochondria from Cd^{2+}-cultured cells. The Cd^{2+} content in mitochondria isolated from Cd^{2+}-cultured cells was 32 ± 14 nmol (mg protein)$^{-1}$ (n=12).

Antioxidant enzymes and metabolites

The increase in the Cu and Fe content in Cd^{2+}-stressed cells suggested an enhanced oxidative stress since these elements are catalysts of the Fenton reaction. This finding was also in agreement with several reports [4, 13], including those for *E. gracilis* [17, 18], in which ROS production increased in the presence of Cd^{2+}. Therefore,

the response of the antioxidant system present in this protist was assessed by determining some of the enzymes and metabolites involved. Activity of catalase was not detected either by polarography or by reaction with hydrogen peroxide in a native gel by using $K_3Fe(CN)_6$ and $FeCl_2$ reaction as contrast stain (data not shown). However, there was a significant increase in the activities of APx, GPx and GR (48, 35 and 56 %, respectively) in Cd^{2+}-stressed cells (Fig. 1). As internal control, the activity of the cytosolic glycolytic enzyme triose phosphate isomerase (TPI) remained constant in both cell types.

The generation of malondialdehyde (MDA), a lipoperoxidation product, was increased 6-fold under 50 µM Cd^{2+} stress (Table 3). In cells exposed to 25 µM Cd^{2+}, the MDA concentration was 2.2 ± 0.9 (5) nmol (mg protein)$^{-1}$, whereas in cells incubated with 10 mM H_2O_2 for 40 min, the MDA concentration was 5.9 and 6.02 nmol (mg protein)$^{-1}$ for control and stressed cells, respectively; this high concentration of H_2O_2 induces cell death [28].

The content and redox state of glutathione was also determined. The results showed a moderate diminution in the reduction level of the glutathione pool due to Cd^{2+} exposure. This was accompanied by an increase in the glutathione pool of approximately 3 times (Table 3). Thus, Cd^{2+} exposure was associated with a more oxidized glutathione redox state. Also, an increased ubiquinone pool under Cd^{2+} stress was found (from 0.4 in control mitochondria to 0.8 nmol (mg protein)$^{-1}$ (n=2) in mitochondria isolated from Cd^{2+}-exposed cells); cold and cyanide stress also induces a similar change in the ubiquinone pool [28].

The synthesis of phytochelatins was induced by Cd^{2+} (data not shown), in agreement with a previous report [20]. The cysteine content was 0.43 ± 0.08 nmol/10^7 cells (n=3) in Cd^{2+}-exposed cells and 0.33 ± 0.04 nmol/10^7 cells (n=3) in control cells, also in agreement with a previous work [28]

Mitochondrial metabolism

To evaluate the effect of Cd^{2+} on mitochondrial metabolism the activities of oxidative stress-sensitive aconitase and several respiratory enzymes were determined. Little is known about aconitase *in E. gracilis* [22]. A *Km* value of 0.8 mM for citrate was determined for both control and stressed mitochondria. Therefore, to evaluate the content of *active* aconitase, the activity was assayed under *Vm* conditions: saturation by substrate (10 mM citrate) and in the absence of products. As expected, in mitochondria isolated from Cd^{2+}-exposed cells the aconitase activity was severely depressed (Table 4).

Similarly, the activities of respiratory complexes III and IV were decreased by 50-60 % under Cd^{2+} exposure. This result correlated well with a diminution from 137.6 ± 12 to 52.5 ± 8 in *a*-type cytochrome and from 74.8 ± 5 to 28.7 ± 8 pmol (mg protein)$^{-1}$ in *b*- type cytochrome in control and Cd^{2+}-exposed mitochondria. In contrast, Cd^{2+} stress had no effect on L-iLDH whereas SDH and D-iLDH decreased by 50 and 40 %, respectively (Table 4).

In good agreement with the observation in intact cells (Table 1), the AOX capacity was 4.5 times higher in mitochondria from Cd^{2+}-exposed cells, accounting for 91 ± 2.6 % of total respiration (Table 4). Furthermore, a correlation between the concentration of $CdCl_2$ in the growth medium and the mitochondrial AOX capacity (Fig 1A) and content (Fig 1B) was observed.

In addition to inducing oxidative stress, Cd^{2+} may also affect the respiratory chain [40]. Then, the direct effect of Cd^{2+} on respiratory enzymes was also evaluated (Fig. 3 and Table 5). Both in mitochondria isolated from control and Cd^{2+}-treated cells, the most sensitive enzyme to Cd^{2+} was the TMPD oxidase, whereas CN^--sensitive L-lactate respiration (L-iLDH + cytochrome complex b-c_1 + cytochrome c oxidase) showed an

intermediate sensitivity. In contrast, the AOX activity was rather resistant to Cd^{2+} (Fig. 3 and Table 5). Thus, this inherent resistance to Cd^{2+}, together with the enhanced AOX content and activity induced by Cd^{2+} exposure, indicate a relevant role for AOX in establishing a protective mechanism against heavy metal-induced oxidative stress.

Discussion

E. gracilis is a Cd^{2+}-resistant organism under a variety of conditions [15, 19, 20]. When growth in glutamate + malate medium was evaluated, the IC_{50} for Cd^{2+} was high and similar to that previously reported for *E. gracilis* cultured with lactate as carbon source [20]. The rate of cellular respiration showed a lower sensitivity to cyanide in Cd^{2+}-cultured cells. This result suggested an increased participation of AOX activity in total respiration. As a consequence, there was a diminished ATP concentration maybe because AOX activity does not participate in oxidative phosphorylation. Moreover, the lowered content of ADP, F-1,6-BP and trioses phosphate in Cd^{2+}-exposed cells suggested inhibition of adenylate kinase (ADP), phosphofructokinase (F-1,6-BP) and aldolase (trioses phosphate), and hence possible diminution in glycolysis.

Cadmium promoted in *E. gracilis* an increased content of iron and cooper, which induce oxidative stress [4]. The mechanism by which these metals are increased in cells exposed to Cd^{2+} is unknown, but an increase of the transporters involved or disturbances of the chelating mechanisms might be responsible for these results. On the other hand, the nutritional status of leaves and roots from pea plants is also altered by Cd^{2+} stress, but under which the metal uptake in both tissues and Fe content in leaves decrease: inhibition or change in selectivity of different transporters was suggested [13]. Iron deficiency under Cd^{2+} stress is also observed in sugar beet [41]. These contrasting results between *Euglena* and plants suggest that different heavy metal homeostatic mechanisms operate.

Increased lipid peroxidation and marked inhibition of some antioxidant enzymes such as catalase and superoxide dismutase was documented for Cd^{2+}-exposed pea plants [13]. Likewise, it has been described for both photosynthetic and heterotrophic *E. gracilis* cells an increased production of ROS and mitochondrial DNA breaks induced by Cd^{2+} exposure [17, 18], which suggests that mitochondria is a primary target of Cd^{2+} in this microorganism.

The lower zinc content in Cd^{2+}-exposed cells (cf. Table 2) may be explained by the well-described inhibitory, competitive effect of zinc on Cd^{2+} transport in photosynthetic *E. gracilis* [19]. Competition between zinc and Cd^{2+} has also been described for mammalian and plant models [42-44].

The antioxidant defense is certainly one of the cellular responses involved in dealing with heavy-metal induced damage [13, 45-48]. However, the lack of some of the most common and relevant defense enzymes in *E. gracilis* seems a risk factor for this protist, making it more sensitive to oxidative stress. Notwithstanding, *Euglena* supports well different stressful conditions such as profound variations in temperature, pH and O_2 concentration that usually lead to severe oxidative stress [28, 33, 49]. This behavior in *Euglena* suggests the presence of mechanisms that compensate the deficiency of some anti-oxidant enzymes to contend with oxidative stress.

The oxidative stress induced by Cd^{2+} and its appropriate management in *E. gracilis* was apparent by (a) the enhancement in the MDA content; (b) the diminution in the level of reduced glutathione; (c) the diminution in the activity of aconitase; (d) the increase in the activity of the anti-oxidant enzymes APx, GPx and GR, which may stimulate the ascorbate-glutathione cycle.

Another relevant aspect of *E. gracilis* is the presence of a branched respiratory chain in its mitochondria. A ramified electron transfer allows organisms to adapt to different environmental conditions [50-52]. In *E. gracilis*, the presence of AOX may

reduce the generation of oxidative stress by draining electrons from an inhibited respiratory chain. Indeed, Cd^{2+} exposure inhibited the activity of the respiratory complexes III and IV, whereas AOX activity enhanced and showed resistance to a direct exposure of Cd^{2+}. Thus, the increased AOX capacity under Cd^{2+} stress apparently promoted an adequate electron flux thus limiting ROS production.

The role of AOX in limiting ROS production has been extensively studied but the present work emphasizes a protective role for AOX under heavy metal stress. Moreover, the present findings contribute to better understand the cellular and mitochondrial mechanisms used by this microorganism to resist and accumulate heavy metals [14-16, 19, 20, 53].

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References

1. M. Valko, H. Morris, M.T. Cronin, Metals, toxicity and oxidative stress, Curr. Med. Chem. 12 (2005) 1161-1208.
2. A. Schützendübel, A. Polle, Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization, J. Exp. Bot. 53 (2002) 1351-1365.

3. N. Ercal, H. Gurer-Orhan, N. Aykin-Burns, Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage, Curr. Top. Med. Chem. 1(2001) 529-539.

4. L. Patrick, Toxic metals and antoxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity, Altern. Med. Rev. 8 (2003) 106-128.

5. A. Hartwig, Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function, Antioxid. Redox Signal. 3 (2001) 625-634.

6. M. Asmuss, L.H.F. Mullenders, A. Hartwig, Interference by toxic metal compounds, with isolated zinc finger DNA repair proteins, Toxicol. Lett 112-113 (2000) 227-231.

7. E. Chavez, R. Briones, B. Michel, C. Bravo, D Jay, Evidence for the involvement of dithiol groups in mitochondrial calcium transport: studies with cadmium, Arch. Biochem. Biophys. 242 (1985) 493-497.

8. F. Petrini, M.A. Iannelli, S. Pasqualini, A. Massacci, Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplast of *Phragmites australis* (Cav.) Trin. ex Steudel, Plant Physiol. 133 (2003) 829-837.

9. P. Faller, K. Kienzler, A. Krieger-Liszkay, Mechanism of Cd^{2+} toxicity: inhibits photoactivation of photosystem II by competitive binding to the essential Ca^{2+} site, Biochim. Biophys. Acta 1706 (2005) 158-164.

10. L.F. De Filippis, H. Ziegler, Effect of sublethal concentrations of zinc, cadmium and mercury on the photosynthetic carbon reduction cycle of *Euglena*, J. Plant Physiol, 142 (1993) 167-172.

11. F. Jemal, M. Zarrouk, M.H. Ghorbal, Effect of cadmium on lipid composition of pepper, Biochem. Soc. Trans. 28 (2000) 907-910.

12. D.C. Ramírez, M.S. Gimenez, Lipid modification in mouse peritoneal macrophages after chronic cadmium exposure, Toxicology 172 (2002) 1-12.

13. L.M. Sandalio, H.C. Dalurzo, M. Gómez, M.C. Romero-Puertas, L.A. del Rio, Cadmium-induced changes in the growth and oxidative metabolism of pea plants, J. Exp. Bot. 52 (2001) 2115-2126.

14. L. Navarro, M.E. Torres-Márquez, S. González-Moreno, S. Devars, R. Hernández, R. Moreno-Sánchez, Comparison of physiological changes in *Euglena gracilis* during exposure to heavy metals of heterotrophic and autotrophic cells, Comp. Biochem. Physiol. 116C (1997) 265-272.

15. S. Devars, C. Avilés, C. Cervantes, R. Moreno-Sánchez, Mercury uptake and removal by *Euglena gracilis*, Arch. Microbiol. 174 (2000) 175-180.

16. 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.

17. M. Watanabe, T. Suzuki, Involvement of reactive oxygen stress in cadmium-induced cellular damage in *Euglena gracilis*, Comp. Biochem. Physiol. C 131 (2002) 491-500.

18. M. Watanabe, K. Henmi, K. Ogawa, T. Suzuki, Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic strains of *Euglena gracilis*, Comp. Biochem. Physiol. C 134 (2003) 227-234.

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

20. C. Avilés, H. Loza-Tavera, N. Terry, R. Moreno-Sánchez, Mercury pretreatment selects an enhanced cadmium-accumulating phenotype in *Euglena gracilis*, Arch. Microbiol. 180 (2003) 1-10.

21. A. Uribe, E. Chavez, M. Jimenez, C. Zazueta, R. Moreno-Sanchez, Characterization of Ca^{2+} transport in *Euglena gracilis* mitochondria, Biochim. Biophys. Acta 1186 (1994) 107-116.

22. S. Kitaoka, Y. Nakano, K .Miyatake, A. Yokota, Enzymes and their functional location: enzymes of oxygen metabolism, in: D.E. Buetow (Ed.), The biology of *Euglena*, vol. IV, Academic Press, New York, 1989, p. 20-91.

23. S. Kanematsu, K. Asada, Ferric and manganic superoxide dismutases in *Euglena gracilis*, Arch. Biochem. Biophys. 195 (1979) 535-545.

24. J.M. Overbaugh, Initial observations on the role of glutathione peroxidases in *Euglena*, J. Free Radic. Biol. Med. 1(1985) 187-193.

25. S. Shigeoka, T. Onishi, Y. Nakano, S. Kitaoka, Characterization and physiological function of glutathione reductase in *Euglena gracilis* Z, Biochem. J. 242 (1987) 511-515.

26. S. Shigeoka, I. Nakano, S. Kitaoka, Metabolism of hydrogen peroxide in *Euglena gracilis* Z by L-ascorbic acid peroxidase, Biochem. J. 186 (1980) 377-380.

27. F. Montrichard, F. Le Guen, D.L. Laval- Martin, E. Davioud-Charvet, Evidence for the co-existence of glutathione reductase and trypanothione reductase in the non-trypanosomatid Euglenozoa: *Euglena gracilis* Z, FEBS Lett. 442 (1999) 29-33.

28. N.A. Castro-Guerrero, K. Krab, R. Moreno-Sánchez, The alternative respiratory pathway of *Euglena* mitochondria, J. Bioenerg. Biomembr. 36 (2004) 459-469.

29. L. Patrick, Toxic metals and antoxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity, Altern. Med. Rev. 8 (2003) 106-128.

30. C.L. Greenblatt, J.A. Schiff, A pheophytin-like pigment in dark-adapted *Euglena gracilis*, J. Protozool. (1959) 23-28.

31. H.U. Bergmeyer, Methods of enzymatic analysis, (Ed.) Verlag Chemie, Wheinheim (1974).

32. J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol. 52 (1972) 302-310.

33. N.A. Castro-Guerrero, R. Jasso-Chávez, R. Moreno-Sánchez, Physiological role of rhodoquinone in *Euglena gracilis* mitochondria, Biochim. Biophys. Acta 1710 (2005) 113-121.

34. T. J. Shoenmakers, G. F. Visser, A. P. R. Theuvenet, CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. Biotechniques 12 (1992) 870-879.

35. A. Hausladen, I. Fridovich, Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not, J. Biol. Chem. 269 (1994) 29405-29408.

36. J.M. Overbaugh, R. Fall, Characterization of a selenium-independent glutathione peroxidase from *Euglena gracilis*, Plant Physiol. 77 (1985) 437-442.

37. Y. Nakano, K. Asada, Hydrogen peroxide is scanvenged by ascorbate-specific peroxidase in spinach chloroplasts, Plant Cell. Physiol. 22 (1981) 867-880.

38. S. Shigeoka, Y. Nakano, S. Kitaoka, Purification and some properties of L-ascorbic-acid-specific peroxidase in *Euglena gracilis* Z, Arch. Biochem. Biophys. 201(1980) 121-127.

39. L.M. Sillen, A.E. Martell, Stability constants of metal-ion complexes, Spec. Publ. - Chem. Soc. 25 (1973) 865 pp.

40. Y. Wang, J. Fang, S.S. Leonard, K.M. Rao, Cadmium inhibits the electron transfer chain and induces reactive oxygen species, Free Rad. Biol. Med. 36 (2004) 1434-1443.

41. M. Greger, S. Lindberg, Effects of Cd^{2+} and EDTA on young sugar beets (*Beta vulgaris*) II Net uptake and distribution of Mg^{2+}, Ca^{2+} and Fe^{2+}/Fe^{3+}, Physiol. Plant. 69 (1987) 81-86.

42. B.S. Kingsley, J.M. Frazier, Cadmium transport in isolated perfused rat liver: zinc-cadmium competition, Am. J. Physiol. 236 (1979) 139-143.

43. H.S. Yu, S.T. Chan, Zinc amelioration of cadmium toxicity on preimplantation mouse zygotes in vitro, Teratology 37(1988) 13-19.

44. J.J. Hart, R.M. Welch, W.A. Norvall, J.M. Clarke, L.V. Kochian, Zinc effects on cadmium accumulation and portioning in near-isogenic lines of durum wheat that differ in grain cadmium concentration, New Phytol. 167 (2005) 391-401.

45. K. Vido, D. Spector, G. Lagniel, S. Lopez, M.B. Toledano, J. Labarre, A proteoma analysis of the cadmium response in *Saccharomyces cerevisiae*, J. Biol. Chem. 276 (2001) 8469-8474.

46. A. Malecka, W. Jarmuszkiewicz, B. Tomaszewska, Antioxidative defense to lead stress in subcellular compartments of pea root cells, Acta Biochim. Pol. 48 (2001) 687-698.

47. A. Guelfi, R.A. Azevedo, P.J. Lea, S.M. Molina, Growth inhibition of the filamentous *Aspergillus nidulans* by cadmium: an antioxidant enzyme approach, J. Gen. Appl. Microbiol. 49 (2003) 63-73.

48. C. Casiot, O. Bruneel, J.C. Personné, M. Leblanc, F.E. Elbaz-Poulichet, Arsenic oxidation and bioaccumulation by the acidophilic protozoan, *Euglena mutabilis*, in acid mine drainage (Carnoules, France). Sci. Total Environ. 320 (2004) 259-267.

49. S. Srivastava, R.D. Tripathi, U.N. Dwivedi, Synthesis of phytochelatins and modulation of antioxidants in response to cadmium stress in *Cucuta reflexa*- an angiospermic parasite, J. Plant. Physiol. 161 (2004) 665-674.

50. J.W. de Gier, M. Lübben, W.N.M.Reijnders, C.A.Tipker, D.J. Slotboom, R.J.M. van Spanning, A.H. Stouthamer, J. van der Oost, The terminal oxidases of *Paracoccus denitrificans*, Mol. Microbiol. 13 (1994) 183-196.

51. R.K. Poole, G.M. Cook, Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation, Adv. Microbiol. Physiol. 43 (2000) 165-224.

52. E.J. Helmerhorst, M.P. Murphy, R.F. Troxler, F.G. Oppenheim, Characterization of the mitochondrial respiratory pathways in *Candida albicans*, Biochim. Biophys. Acta 1556 (2002) 73-80.

53. D.G. Mendoza-Cozatl, E. Rangel-Gonzalez, R. Moreno-Sanchez, Simultaneous Cd^{2+}, Zn^{2+} and Pb^{2+} uptake and accumulation by photosynthetic *Euglena gracilis*, Arch. Environ. Contam. Toxicol. 51 (2006) 521-528.

Figure legends

Figure 1. Effect of 50 µM $CdCl_2$ on antioxidant enzyme activities

APx: ascorbate peroxidase; GPx: glutathione peroxidase; GR: glutathione reductase; TPI: triose phosphate isomerase. * Units of triose phosphate isomerase are given as U/ mg protein.

Figure 2. Effect of Cd^{2+} on mitochondrial respiration and AOX

A: Effect of varying the $CdCl_2$ concentration on the mitochondrial respiratory activity and AOX capacity. 100% was considered as the rate of respiration with 10 mM L-lactate as oxidizable substrate and in the absence of inhibitors. **B**: Western blot of *E. gracilis* mitochondrial preparations (50 µg protein) from control cells and exposed to 50 µM $CdCl_2$. Dilutions of antibodies were 1:9000 and 1:500 for AOX and ANT respectively.

Figure 3. Inhibition by Cd^{2+} of respiratory enzymes

A: Mitochondria isolated from control cells. ▲, TMPD oxidase activity; ■, L-lactate oxidase; ●, AOX activity. **B**, Mitochondria isolated from *Euglena* cells cultivated in the presence of 50 µM Cd^{2+}. O, TMPD oxidase activity; □, AOX activity. Insets show a zoom of the region below 100 µM Cd^{2+}. Enzyme activities and free Cd^{2+} concentrations were determined as indicated under the Materials and Methods. The protein concentration used in the activity assays was 1.7-2.5 mg for L-lactate oxidase and AOX (for mitochondria from control cells) and 0.5-0.8 mg for AOX assays (for mitochondria from Cd^{2+}-treated cells), while 0.4-0.8 mg of protein was used for TMPD oxidase assays for both types of mitochondria. Results are representative of 4 different mitochondrial preparations. Maximal activities and IC_{50} values are shown in Tables 4 and 5.

Table 1. Effect of Cd^{2+} on growth, respiration and high-energy related phosphate metabolites in *E. gracilis* cells

	Z	ZCd_{50}
cell density at 96 h (X 10^6 cell/ ml)	7.3 ± 0.2	1.3 ± 0.1[a]
Cellular respiration ng at O(min x 10^7 cell)$^{-1}$	110 ± 7	113 ± 6[ns]
+ 1 mM cyanide	20 ± 8	78 ± 3[a]
ATP (mM)	0.96 ± 0.08	0.69 ± 0.27[ns]
PPi (mM)	0.76 ± 0.12	1.09 ± 0.21[ns]
ADP (mM)	2.04 ± 0.25 (5)	0.76 ± 0.06 (5)[a]
AMP (mM)	0.99 ± 0.37	1.09 ± 0.2[ns]
G6P (mM)	4.8 ± 4	3.9 ± 1[ns]
F6P (mM)	1.46 ± 0.96	1.05 ± 0.36[ns]
F1,6BP (mM)	0.33 ± 0.16 (5)	0.17 ± 0.03 (9)[a]
Trioses phosphate (mM)	0.83 ± 0.1	0.28 ± 0.07 (7)[a]

Values are mean ± SD of three independent preparations except where is otherwise indicated. [ns]: not significantly different versus Z; [a]: P< 0.005 versus Z (Student t-test for non-paired samples). Intracellular water volume used for calculations was 2.4 and 6.3 µL (10^7 cell)$^{-1}$ for control (Z) and Cd^{2+}-stressed cells (ZCd_{50}), respectively [20]

Table 2. Trace element content in culture medium and in *E. gracilis* cells (nmol (10^7 cells)$^{-1}$)

	Cd	Mn	Fe	Co	Ni	Cu	Zn	K	Mg	Ca
Culture medium (µM)	51.7 ± 4	464 ± 9	36 ± 3.6	12.8 ± 5	6.4 ± 1.6	9.6 ± 1.3	468 ± 38	320 ± 20	770 ± 65	248 ± 39
Z cells	0 (29)	46 ± 3 (10)	15 ± 3 (13)	4.6 ± 0.6 (10)	1.2 ± 0.3 (6)	0.6 ± 0.3 (10)	200 ± 9 (7)	156 ± 27 (7)	675 ± 190 (7)	18.6 ± 12 (10)
ZCd_{50} cells	86.3 ± 25[a] (27)	70 ± 3[a] (10)	48 ± 9[a] (12)	9.4 ± 1.5[a] (13)	1.6 ± 0.6[ns] (6)	4.1 ± 0.6[a] (13)	110 ± 3[a] (7)	242 ± 60[ns] (27)	270 ± 70[a] (11)	6 ± 3[ns] (13)

Values are mean ± SD of the number of experiments reported between parentheses. Determination of the elements was carried out with control cells and cells exposed to 50 µM $CdCl_2$. Cells were washed with an EGTA buffer. [ns]: not significantly different versus Z; [a]: P< 0.005 versus Z (Student t-test for non-paired samples).

.

Table 3. Effect of Cd^{2+} exposure on MDA and glutathione content in *E. gracilis cells*

	MDA	GSH	GSH +GSSG
	nmol $(mg)^{-1}$	%	nmol $(10^7$ cells$)^{-1}$
Z	0.5 ± 0.1 (7)	88 ± 2.6 (3)	6.4 ± 0.7 (3)
Z_{Cd50}	3 ± 0.4 (8)[a]	69 ± 9 (3)[a]	18 ± 0.6 (3)[a]

Values are mean ± SD of the number of experiments reported between parentheses.

[a]: P< 0.005 versus Z; (Student t-test for non-paired samples).

Table 4. Effect of Cd^{2+} on mitochondrial enzyme activities

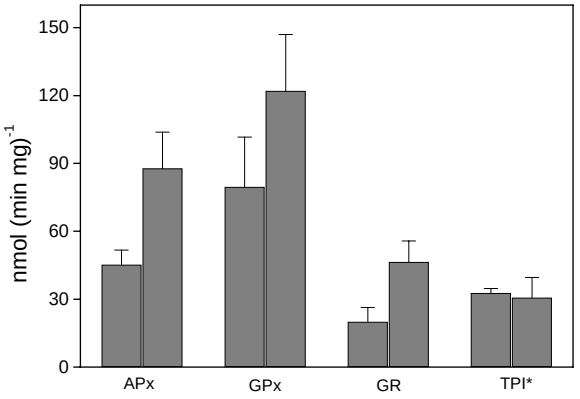
Enzyme	Z	ZCd50
Aconitase	1066 ± 270 (6)	326 ± 118 (7)[a]
L-iLDH	274 ± 71 (5)	237 ± 15 (3)[ns]
complex III	95 ± 11 (4)	41 ± 7 (3)[a]
complex IV	404 ± 69 (8)	160 ± 62 (4)[a]
AOX	35 ± 6 (8)	156 ± 37 (8)[a]

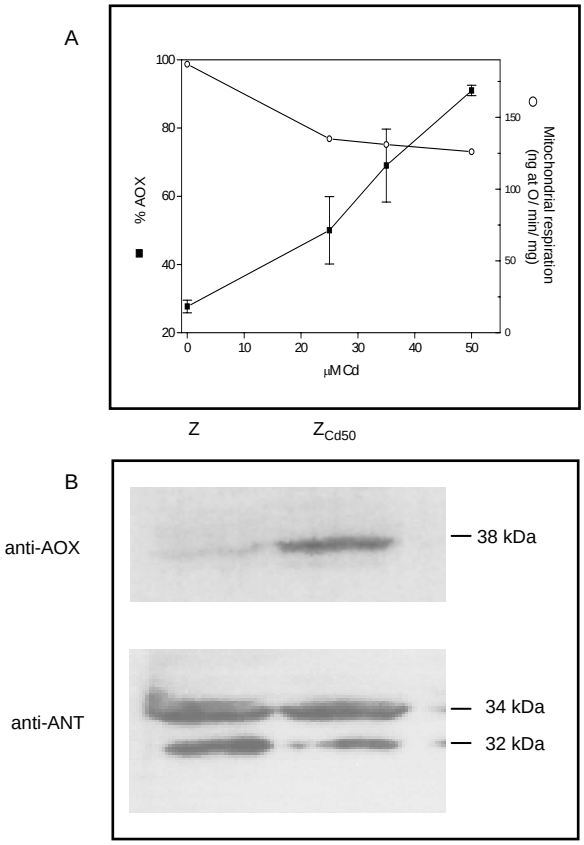
Values are mean ± SD of the number of experiments reported between parentheses. Enzyme activities were performed at 25 °C. Activity of aconitase is given as nmol (min mg)$^{-1}$, L-iLDH activity units are nmol DCPIP (min mg)$^{-1}$, complex III activity units are nmol cyt c red (min mg)$^{-1}$ and complex IV and AOX activities units are ng atoms oxygen (min mg)$^{-1}$. Total rates for mitochondrial respiration with 10 mM L-lactate as substrate were: 170 ± 50 (n= 8) and 72 ± 18 (n=11) ng atoms oxygen (min x mg protein)$^{-1}$ for control and Cd^{2+}-stressed cells, respectively. AOX capacity was evaluated in the presence of 10 mM L-lactate and 1 mM cyanide. [ns]: not significantly different; [a]: P< 0.005 versus Z..

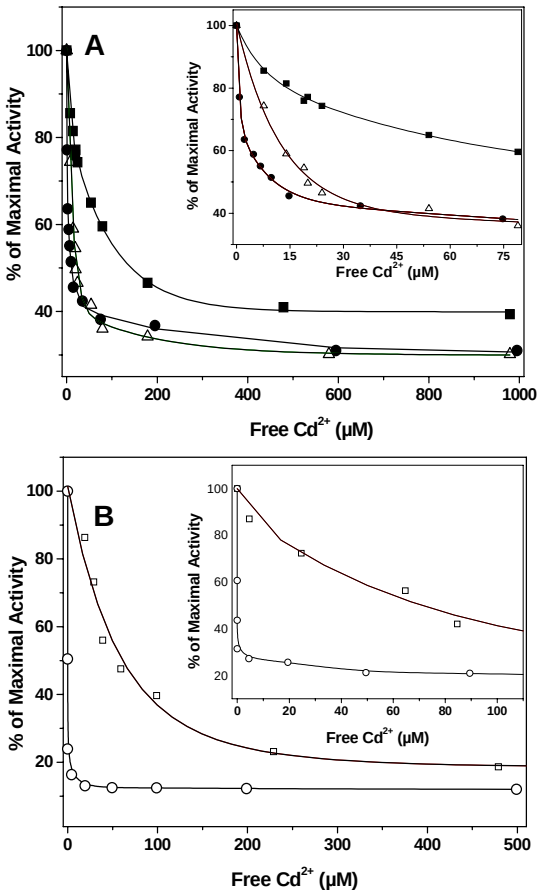
Table 5. Sensitivity of respiratory enzymes to Cd^{2+} in mitochondria isolated from control and Cd^{2+}-treated cells.

	IC_{50} (μM)	
	Control	**Cd^{2+} mitochondria**
L-Lactate oxidase	14 ± 4 (n=4)	
AOX	38 ± 11 (n=4)	34 ± 10 (n=5)
TMPD Oxidase	3.7 ± 1.7 (n=4)	0.21 ± 0.2 (n=4)

For these experiments, mitochondria were incubated in 120 mM KCl, 20 mM MOPS, pH 7.25 (an EGTA-lacking buffer). The free Cd^{2+} concentration was calculated by using the software CHELATOR [34]. Enzyme activities were measured as detailed under Materials and Methods. The IC_{50} values were estimated from titration curves like those shown in Fig. 3. For L-lactate oxidase in mitochondria isolated from cells exposed to Cd^{2+}, the Cd^{2+} sensitivity was not determined due to the low activity of this respiratory component and the consequent inaccuracy of the measurements.


150
120
90
60
30
0
nmol (min mg)-1
APx
GPx
GR
TPI*


A
100
80
60
40
20
% AOX
0
10
20
30
40
50
µM Cd
150
100
50
0
Mitochondrial respiration
(ng at O/ min/ mg)
Z
Z Cd50
B
anti-AOX
38 kDa
anti-ANT
34 kDa
32 kDa


A
% of Maximal Activity
Free Cd2+ (μM)
B
% of Maximal Activity
Free Cd2+ (μM)

DATOS NO MOSTRADOS EN EL ARTÍCULO.

(**1**) Detección de tripanotión en *E. gracilis*.

Como se mencionó en la sección introductoria, el tripanotión es un conjugado de glutatión-espermidina que funciona como sustrato en diversas reacciones metabólicas. Se desconoce la presencia de este compuesto en *E. gracilis*. Sin embargo, existe un reporte en este protista donde se analizó y detectó la presencia de una proteína similar a la tripanotión reductasa presente en tripanosomátidos. Esto sugiere la presencia del tripanotión y de otras enzimas dependientes de este metabolito en *Euglena*. Durante el desarrollo de este trabajo y con base en el uso de estándares comerciales detectamos mediante HPLC la presencia de un compuesto tiolado que correspondió al tiempo de retención del estándar de tripanotión. Sin embargo, el estándar originó dos picos en el cromatograma correspondientes a compuestos diferentes (7.9 min y 18 min). Ambos fueron evidenciados en extractos ácidos de *E. gracilis*. Los compuestos del estándar y de las muestras sólo fueron detectados en presencia de un reductor (DTT). Debido a que obtuvimos datos inconsistentes en nuestras muestras los resultados no fueron considerados para la publicación.

En la Fig. 9 se muestra un ejemplo de los cromatogramas obtenidos a partir de un extracto representativo, así como de los estándares utilizados. Se evidenciaron compuestos a tiempos de retención más largos que no correspondieron a ninguno de los estándares utilizados, de manera que su naturaleza es desconocida hasta este momento.

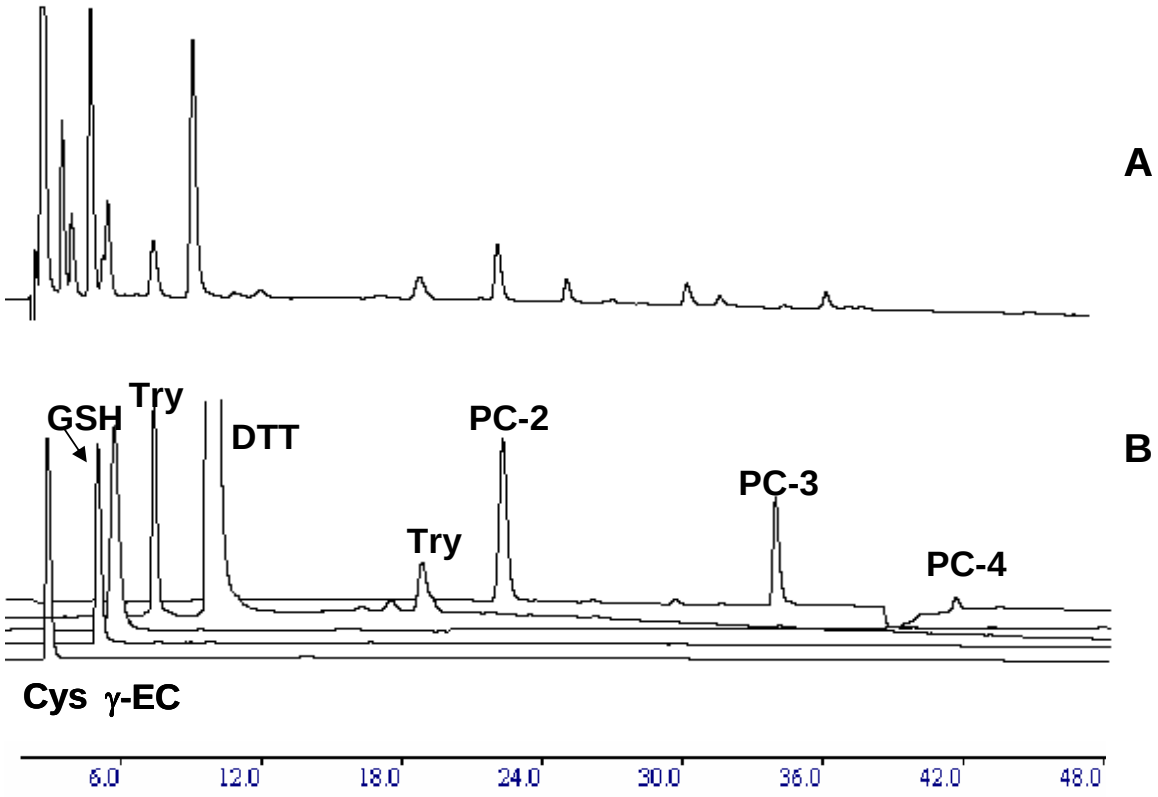


Fig. 9.- Cromatogramas representativos obtenidos por HPLC a 412 nm. (A) Extracto citosólico de *E. gracilis* heterotrófica; (B) estándares comerciales disueltos en agua y añadidos por separado.

(**2**) Estandarización de la determinación de las actividades de la aconitasa y de las enzimas antioxidantes.

En el caso de la aconitasa fue necesario ensayar algunas de las condiciones que son utilizadas para detectar la actividad, puesto que en *E. gracilis* no existe mucha información acerca de esta enzima. En la Fig. 10 se muestran ensayos representativos de esta actividad enzimática, en la que se obtuvieron los valores de *Km* para los diferentes sustratos, así como las condiciones óptimas de medición.

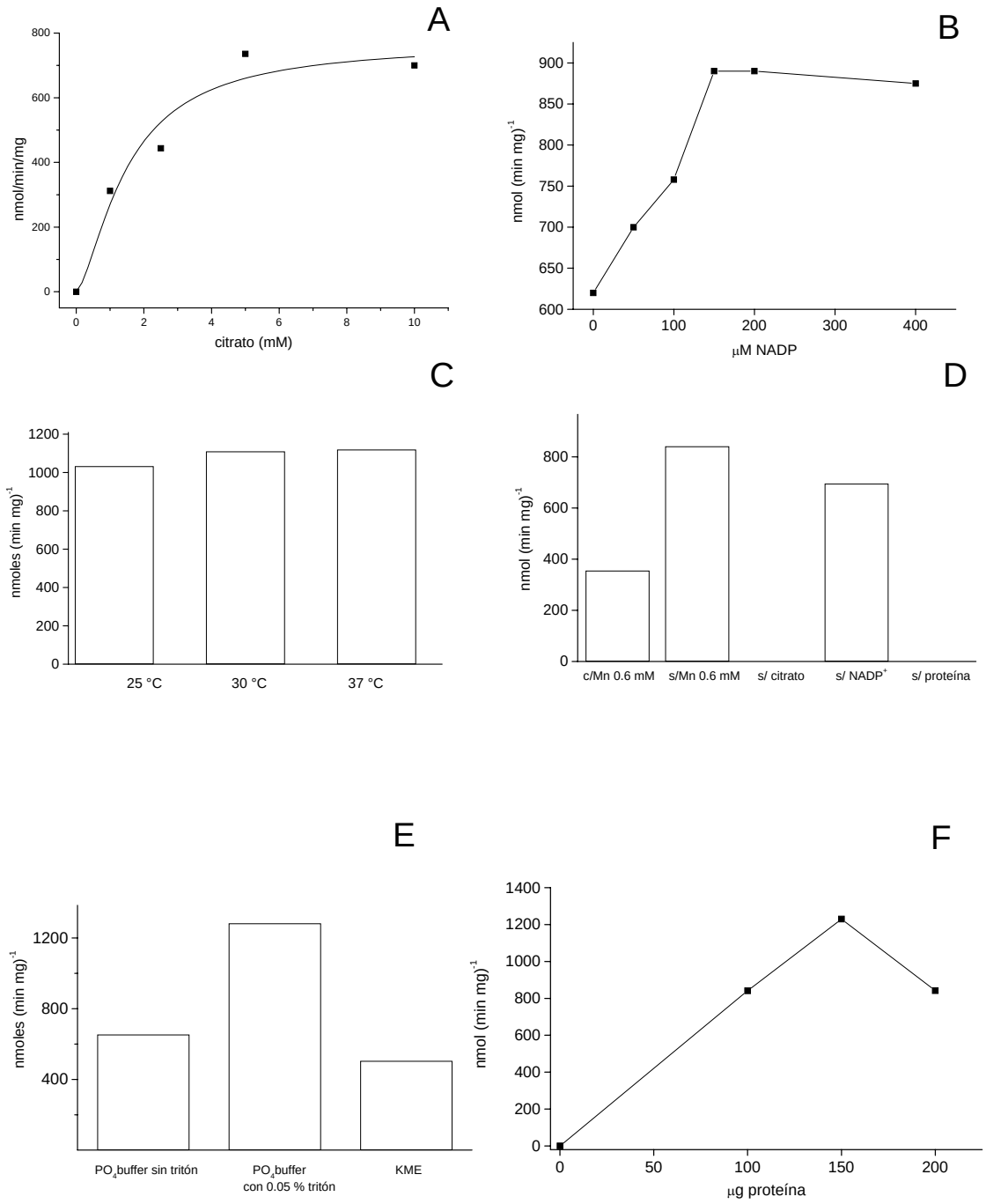


Fig. 10.- Ensayos enzimáticos representativos de la caracterización de la aconitasa en mitocondrias de *E. gracilis*. **A**: Cinética de saturación por citrato utilizando 200 µM de $NADP^+$ a 25 °C en amortiguador de fosfatos con tritón X-100 y 150 µg de proteína mitocondrial; **B**: Dependencia de la actividad sobre la [$NADP^+$] en el ensayo utilizando 10 mM de citrato a 25°C en amortiguador de PO_4 con tritón X-100 y 150 µg de proteína mitocondrial; **C**: Dependencia de la actividad sobre la temperatura; **D**: Controles del ensayo enzimático; **E**: Dependencia de la actividad sobre el buffer de ensayo; **F**: Dependencia de la actividad de la aconitasa sobre la concentración de proteína usada en el ensayo.

(**3)** Para la evaluación de las enzimas antioxidantes se utilizaron las condiciones basadas en los parámetros cinéticos evaluados.

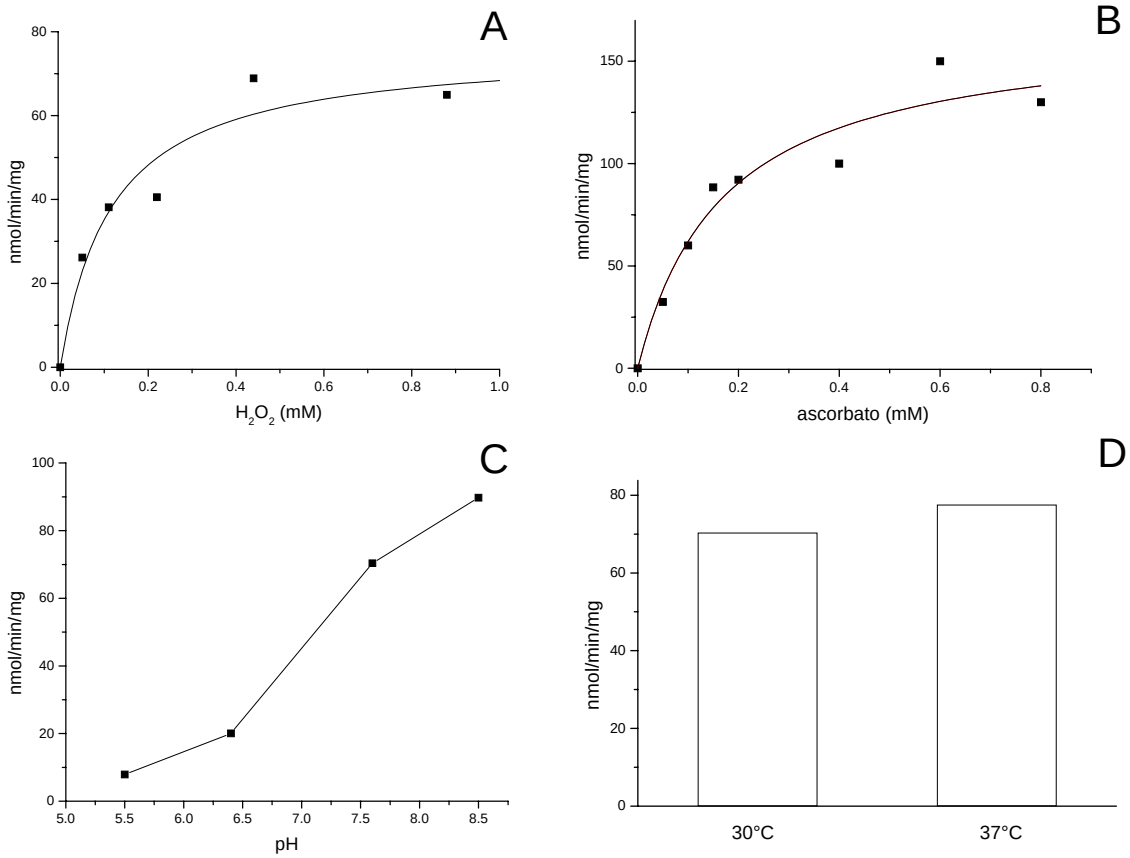


Fig. 11.- Ensayos enzimáticos representativos de la caracterización de la APx en extractos citosólicos de *E. gracilis*. **A**, **B**: Cinéticas de saturación por los sustratos H_2O_2 y ascorbato a 30 °C; la concentración del sustrato fijo fue 2 mM de ascorbato y 2 mM de H_2O_2 respectivamente; **C**: Dependencia de la actividad de la APx sobre el pH; **D**: Dependencia de la actividad de la APx sobre la temperatura.

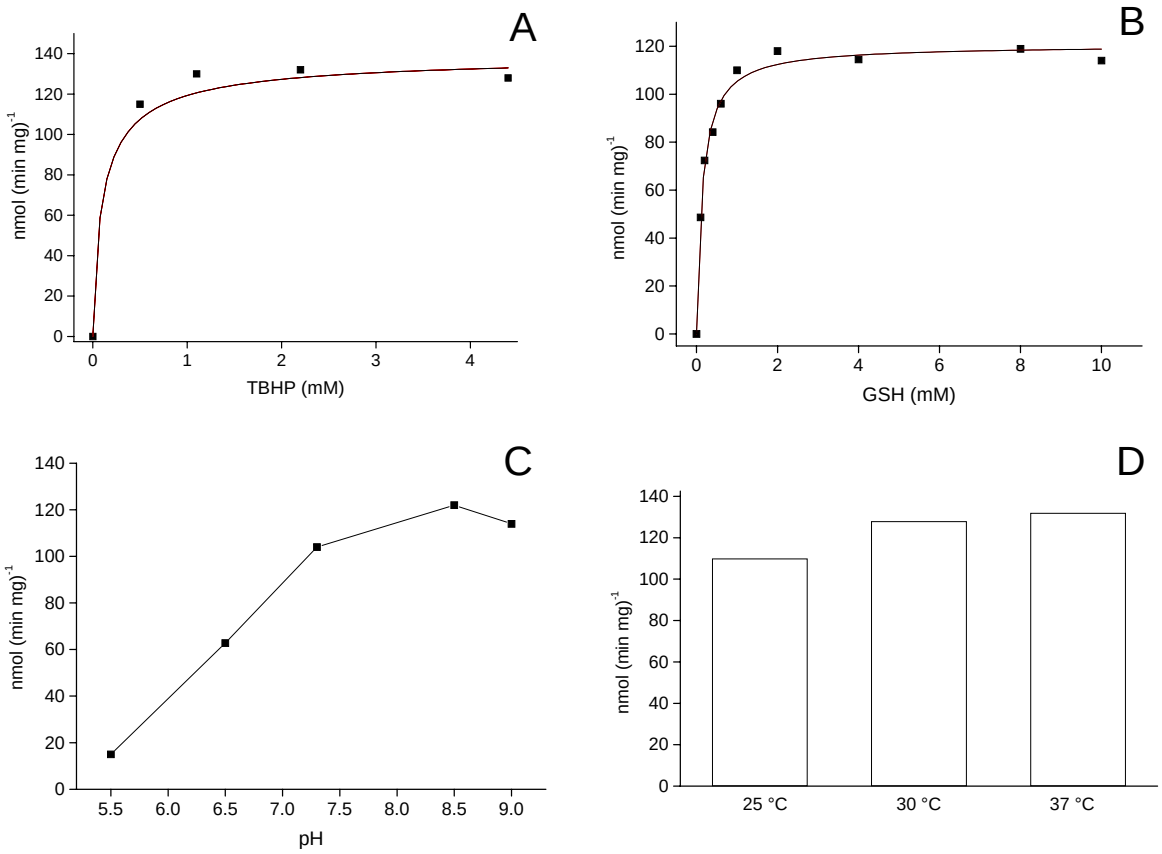


Fig. 12.- Ensayos enzimáticos representativos de la GPx en extractos citosólicos de *E. gracilis*. **A**, **B**: Cinéticas de saturación por los sustratos (TBHP y GSH) realizados a 30 °C usando como sustrato fijo 5 y 1 mM de GSH y TBHP respectivamente; **C**: Dependencia de la actividad de la GPx sobre el pH; **D**: Dependencia de la actividad de la GPx sobre la temperatura.

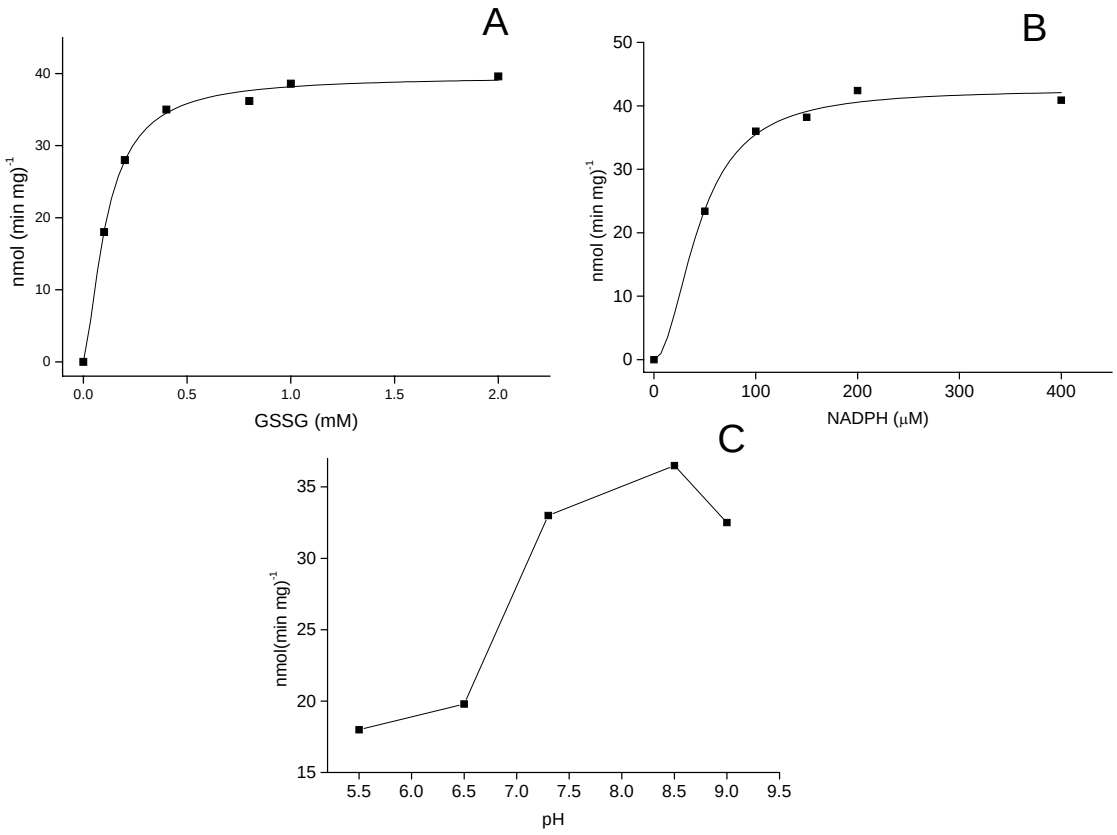
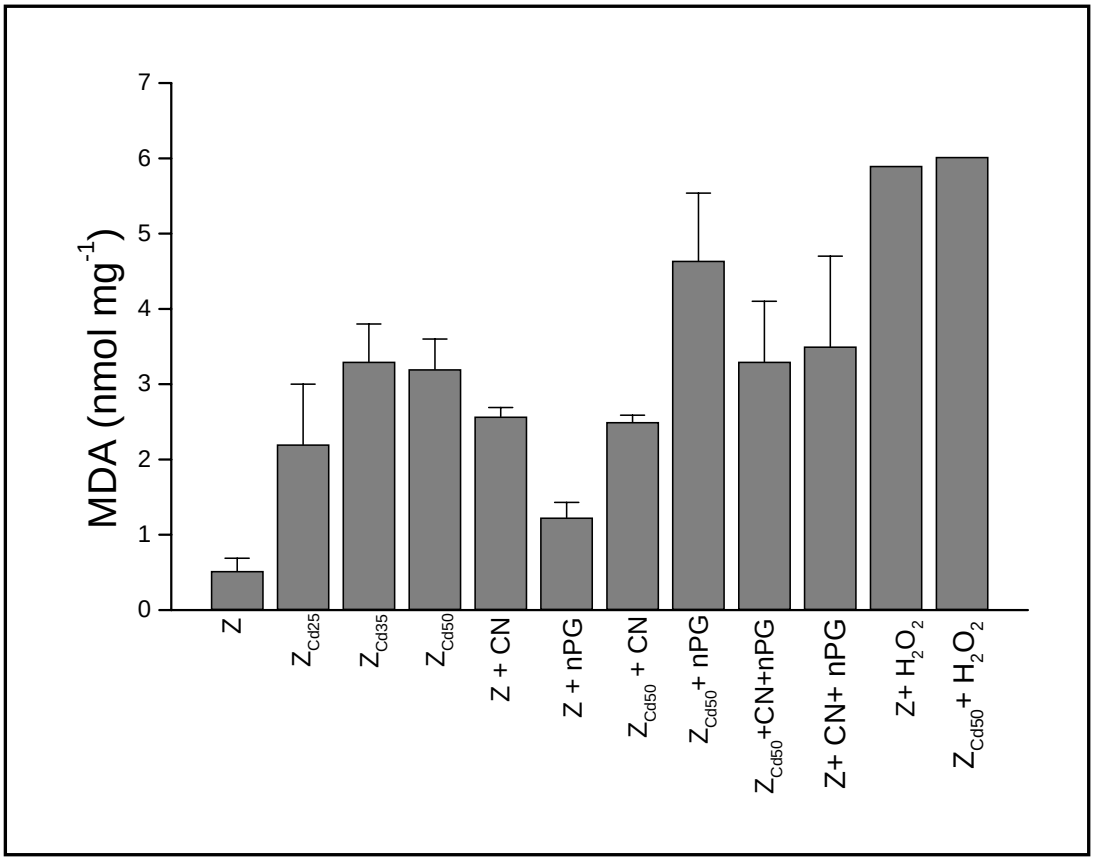


Fig. 13.- Ensayos enzimáticos representativos de la GR en extractos citosólicos de *E. gracilis*. **A**, **B**: Cinéticas de saturación por los sustratos de la GR (GSSG y NADPH) usando como sustrato fijo 200 µM y 1 mM de NADPH y GSSG, respectivamente; **C**: Dependencia de la actividad de la GR sobre el pH.

(**3**) Estrés oxidativo: Determinación de lipoperóxidos

La determinación de lipoperóxidos se realizó mediante la medición de los niveles de malondialdehído (MDA) con el objetivo de evaluar el estrés oxidativo generado. Además de los valores reportados en el artículo, se realizaron experimentos para evaluar lo que sucedió en experimentos en los que se inhibió a la vía clásica con cianuro 1 mM, a la vía alternativa con nPG 3 mM y la inhibición de ambas vías al mismo tiempo. A la par se realizaron controles exponiendo a las células a 10 mM de H_2O_2 por 40 min como un control del máximo contenido del MDA que podría esperarse, debido a que esta concentración de peróxido promueve la muerte celular.



Los resultados mostraron que la exposición a cadmio incrementa la concentración del MDA (mayor estrés oxidativo). La inhibición de la vía clásica en células control y expuestas a cadmio o a alguna otra condición de estrés promueve una concentración del MDA similar, no así la inhibición de la vía alternativa, la cual muestra un claro incremento del MDA en células que están expuestas a cadmio y que poseen una actividad de AOX mayor. Cuando se inhiben ambas vías los valores del MDA son similares en ambos modelos. Los valores máximos del MDA fueron obtenidos con la concentración de peróxido que causa muerte celular en *E. gracilis.*

CAPÍTULO IV

DISCUSION GENERAL

El trabajo realizado en esta tesis demostró que la AOX presente en mitocondrias de *Euglena gracilis* posee algunas semejanzas con las enzimas reportadas en otros organismos eucariontes. Las características evaluadas de sensibilidad a inhibidores, pH óptimo, la ausencia de un grupo hemo atípico y la detección de una banda proteica del peso molecular correspondiente (38 kDa) mediante el uso de un anticuerpo anti-AOX generado contra la proteína del alga *Chlamydomonas reinhardtii,* apoyan fuertemente que la AOX de *E. gracilis* pertenece a la familia de AOX reportada para eucariontes, y por lo tanto no corresponde a las enzimas alternas reportadas para bacterias.

Hasta nuestro estudio, ninguno de los mecanismos regulatorios descritos (estado redox de la enzima, alfa-cetoácidos, AMP ó GMP) para la AOX de eucariontes se han confirmado para la enzima de *Euglena*. En un reporte anterior [Sharpless TK and Butow RA (1970) An inducible alternate terminal oxidase in *Euglena gracilis* mitochondria, J. Biol. Chem. 245: 58-70], se demostró una activación por AMP como se ha propuesto para levaduras, hongos filamentosos y tripanosomátidos. La condición utilizada consistió en la utilización del succinato como fuente de carbono en los medios de cultivo adicionado de antimicina (un inhibidor de la cadena respiratoria clásica).

En el mismo grupo que reportó este resultado y en el nuestro, se observó que hay ausencia de esta activación en mitocondrias provenientes de células cultivadas en medios que contienen al glutamato y al malato como fuentes de carbono. Aún con la adición de un inhibidor respiratorio (el cianuro) no se obtuvo un resultado diferente. Esto podría

sugerir la presencia de una AOX activable que surge con la incubación con succinato y antimicina y que es diferente a la enzima que registramos en nuestros experimentos. Para explorar esta posibilidad habría que realizar experimentos para reproducir estos datos, pero además, no existen estudios acerca del gen o genes de la AOX que en este protista pudieran establecer la presencia de isoenzimas de la AOX en *E. gracilis*.

Un aspecto importante de este trabajo es la dilucidación de los papeles fisiológicos de la AOX en las mitocondrias de *E. gracilis.*

Alternativamente a la participación de la AOX en la producción de calor en plantas termogénicas, se ha evaluado su función en los diferentes estadios de vida de los tripanosomátidos, siendo en la etapa sanguínea, dependiente de la glucólisis, donde la actividad de la AOX aumenta considerablemente. En el otro estadio de vida, cuando existe una cadena respiratoria, los tripanosomátidos utilizan a la AOX como drenador de electrones para evitar la sobrerreducción de los componentes y por lo tanto la generación de estrés oxidativo [45].

Una de las particularidades de *E. gracilis* es su capacidad de adaptarse a ambientes extremos como cambios drásticos de temperatura, de concentraciones de oxígeno y a la presencia de concentraciones tóxicas de metales pesados. Paradójicamente, *E. gracilis* carece de algunos de los sistemas antioxidantes más importantes que existen en la célula, como la actividad de la catalasa o la de las superóxido dismutasas [48, 49]. Nuestra propuesta sugiere que la complejidad de la cadena respiratoria en las mitocondrias de *E. gracilis* le confiere, en parte, la capacidad de crecer en condiciones que generan estrés oxidativo.

Al afectarse negativamente la vía clásica, principalmente a nivel del complejo III, por la exposición a la baja temperatura o al cadmio, el aumento de la actividad y concentración de la AOX apoyaría la función de la enzima de drenar los electrones que se acumulan al estar bloqueada la otra sección de la vía que los consume. La limitada producción de EROs y la participación de los sistemas antioxidantes presentes en la célula, favorecen la supervivencia de *E. gracilis* y la posibilidad de crecer y duplicarse en condiciones adversas. El cadmio produce EROs en *E. gracilis* (Watanabe y cols.) y MDA. La inhibición de la AOX en el modelo estresado aumentaba significativamente la concentración de MDA reflejando la importancia de la vía alternativa para mantener activa la transferencia de electrones. Estos resultados concuerdan con experimentos anteriores en el modelo de estrés por frío, donde la inhibición de la AOX en el medio de cultivo utilizando nPG afectaba negativamente el crecimiento celular.

Por el contrario, encontramos que la condición de baja concentración del oxígeno afecta negativamente a ambas vías (clásica y alternativa). En modelos bacterianos y en nemátodos, la ausencia del oxígeno genera un cambio en la transferencia de electrones en la cadena respiratoria. En esta situación, la fumarato reductasa (FR) participa activamente reduciendo el fumarato a succinato. Si bien detectamos esta actividad en condiciones microaerofílicas en *E. gracilis*, interesantemente la encontramos también en condiciones aeróbicas. Esta enzima fue sensible a inhibidores de la SDH y no sólo eso, la actividad de FR es mayor a la actividad de SDH que hemos registrado en diferentes condiciones. Esto origina varias interrogantes: ¿Cuál es la función de la FR en aerobiosis?, ¿La enzima responsable de la reducción de fumarato es la SDH o se trata de otra enzima? ¿Cuál es la naturaleza de la SDH en *E. gracilis*? Aunque estas preguntas

obviamente no se han contestado, se mostró que la reducción de la rodoquinona por la D-lactato deshidrogenasa mitocondrial es específica y la capacidad *in vitro* que tiene la cadena clásica de oxidar al rodoquinol. Esta conclusión aporta información valiosa y novedosa sobre la especificidad en una red compleja de reacciones y explica la presencia de concentraciones importantes de rodoquinona durante la aerobiosis, mostrando otro posible mecanismo de transferencia de electrones que pudiera vaciar a los electrones acumulados y evitar o atenuar la generación de EROs. Lo que resultó claro en estos experimentos es que la AOX no tiene una función relevante durante la microaerofilia.

Finalmente, el trabajo de esta tesis mostró que existen diversas condiciones extremas a las cuales puede encontrarse expuesta *E. gracilis* y la manera en que éste organismo puede contender con ellas. Aunque no existe una respuesta única y general a las condiciones adversas, como observamos con la exposición a condiciones microaerofílicas, aquellas condiciones que generan estrés oxidativo tienen un mismo denominador en la mitocondria, que es un aumento de la participación de la AOX.

CONCLUSIONES

1.- La exposición a baja temperatura ocasiona estrés oxidativo y uno de los principales sitios afectados en la mitocondria es el complejo III. La reducción de oxígeno a través de la AOX se incrementa. La SDH también incrementó su actividad.

2.- La exposición a microaerofilia afectó la cadena respiratoria aeróbica. Las enzimas más afectadas fueron la L-LDH, SDH y el complejo III. La transferencia de electrones a través de la AOX se afectó de manera similar. Durante este evento la reducción de fumarato se incrementó, siendo la D-iLDH la enzima reductora de la poza de rodoquinona.

3.- Las enzimas respiratorias más afectadas por la exposición a cadmio fueron los complejos III y IV.

4.- El cadmio incrementó el estrés oxidativo (medido como MDA). La respuesta generada correspondió a un incrementó de diversas enzimas antioxidantes y de la AOX en la mitocondria.

CONCLUSION GENERAL

E. gracilis puede tolerar condiciones que generan estrés oxidativo y que inhiben o limitan la vía clásica mediante la actividad de la AOX, la cual drena los electrones acumulados de la poza de quinonas, evitando así la generación de EROs.

PERSPECTIVAS

Las perspectivas que se generan de este trabajo:

1.- Regulación de la AOX: aunque diversos esfuerzos se han realizado en nuestro grupo para evaluar la forma en que la AOX es regulada en *E. gracilis*, no se ha tenido éxito ensayando las diferentes condiciones que se han aplicado en otros organismos. Esto resultaría en un caso aislado de las familias de la AOX descritas, pero aún así, la obtención del gene y su caracterización y posterior análisis estructural revelarían el grado de similitud de esta enzima en *E. grac*ilis con las familias ya reportadas. Asimismo, la purificación de la AOX podría arrojar resultados respecto a algunas propiedades fisicoquímicas que esclarecieran de forma más certera, su naturaleza. Hasta el momento, los esfuerzos iniciales de purificación de esta enzima en *Euglena gracilis* con algunos detergentes como digitonina o lauril maltósido (que han sido utilizados en *T. brucei*) han resultado en preparaciones sin actividad de AOX.

2.- Metabolismo en otras condiciones de estrés: el trabajo realizado hasta el momento explica claramente el papel de la AOX. Sin embargo, existen otras condiciones extremas en las que *E. gracilis* puede sobrevivir, como estrés salino o presencia de otros metales con mecanismos de toxicidad diferentes al cadmio, como el cromo o el mercurio. Estas condiciones podrían involucrar la participación de uno o varios de los componentes alternativos que ya se han identificado en *Euglena*. Además de cambios importantes en la cadena respiratoria, resultaría importante analizar lo que sucede en otras vías metabólicas.

3.- SDH: claramente encontramos un incremento de la actividad de la SDH durante los eventos que generan estrés oxidativo. Además, se detectó la actividad de la fumarato reductasa y ésta fue sensible a inhibidores de la SDH (TFA, malonato y oxaloacetato), lo que sugiere que la actividad de fumarato reductasa puede corresponder a un solo complejo respiratorio que cataliza la reacción de reducción de fumarato u oxidación de succinato según la condición en la cual *E. gracilis* se encuentre. Aunque se sabe que la SDH puede catalizar la reacción reversa, el aspecto más interesante es que en las condiciones de trabajo utilizadas, incluyendo la aerobiosis, la actividad de la FR tuvo valores mayores que la de SDH. La caracterización bioquímica de esta enzima en *E. gracilis* resultaría útil y valiosa para entender su naturaleza.

4.-Tripanotión: poco se sabe del metabolismo de este compuesto en *E. gracilis* y aunque su importancia puede estar poco relacionada con la principal línea de este proyecto, resultados preliminares detectaron la presencia de este compuesto, despertando la interrogante sobre la relevancia del tripanotión en el sistema antioxidante de *Euglena* y por tanto la relación que guarda este protista con los tripanosomátidos, organismos en los cuales existe este compuesto en lugar de glutatión. Aunque en *Euglena* existe una alta concentración de glutatión y existe un conjunto de enzimas que utilizan al glutatión como sustrato, el establecer la presencia de concentraciones significativas de tripanotión y glutatión al mismo tiempo resultaría novedoso.

5.- *By-pass respiratorio tipo III*: este es otro de los componentes alternativos de la cadena respiratoria de *Euglena* que ha sido propuesto con base en resultados mediante el uso de

inhibidores del complejo III. La presencia de un complejo III atípico sensible a antimicina e insensible a mixotiazol y la no existencia de algún grupo hemo diferente a los correspondientes a este complejo (resultados del trabajo de Maestría) han sugerido la existencia de dos poblaciones de complejo III. Sin embargo, no se han realizado experimentos o purificaciones que muestren al responsable de esta actividad alternativa.