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CARACTERIZACIÓN E IDENTIFICACIÓN DE NUEVOS COMPONENTES PROTEICOS MITOCONDRIALES DE LAS ALGAS CHLAMYDOMONADACEAS

T E S I S

que para obtener el grado de
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presenta

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INDICE

E

RESUMEN

El género *Chlamydomonas* es un miembro de la clase Chlorophyceae y contiene más de 600 especies; la gran mayoría de ellas son fotosintéticas, pero también existen algunas algas incoloras incapaces de crecer autotróficamente. Se supone que las algas incoloras, como los miembros de los géneros *Polytoma* y *Polytomella*, han perdido totalmente el aparato fotosintético que alguna vez tuvieron. Otras posibles causas de la pérdida de la fotosíntesis son el estrés selectivo del ambiente y/o la interacción con otros organismos, con los cuales se pudo haber establecido una transferencia lateral de genes.

El alga verde *Chlamydomonas reinhardtii* ha sido utilizada como un modelo biológico para el estudio de la fotosíntesis; en ella, el cloroplasto, como el asiento principal del fenómeno fotosintético, ha sido extensamente estudiado. Por el contrario, las mitocondrias y su papel en el metabolismo de las células fotosintéticas en general y de *C. reinhardtii* en particular, han recibido poca atención. Ya que la luz es uno de los factores externos más importantes para los organismos fotosintéticos, el efecto de la luz sobre la función y la biogénesis mitocondriales es un tema interesante que merece estudiarse con más cuidado. El alga heterotrófica *Polytomella* sp. es un organismo modelo para estudiar las consecuencias de la pérdida de la fotosíntesis en un marco metabólico muy semejante al de *C. reinhardtii*.

Polytomella sp. es un pariente cercano de *C. reinhardtii*, y comparte con ella varias pecularidades en sus complejos respiratorios. Sin embargo, también se han descrito diferencias importantes, que probablemente se deben a la pérdida de un aparato fotosintético funcional. En este trabajo se describen los mayores complejos de la fosforilación oxidativa analizados con un enfoque proteómico, utilizando mitocondrias aisladas de *C. reinhardtii* y de *Polytomella* sp. En ambas algas, la ATP sintasa tiene un carácter dimérico muy estable, y además exhibe algunas subunidades atípicas. Las subunidades de la citocromo *c* oxidasa de *C. reinhardtii* mostraron características extraordinarias (como es la presencia de una subunidad COXII fragmentada). También, sorprendentemente, en las mitocondrias de *Polytomella* sp. se encontró la presencia de una enzima anaeróbica de tipo bacteriano, una aldehído/alcohol deshidrogenasa, así como un gen que codifica a la misma proteína en el genoma de *C. reinhardtii*.

A diferencia de *C. reinhardtii*, *Polytomella* sp. puede crecer en un margen muy amplio de pH, y puede utilizar diferentes fuentes de carbono. Se ha demostrado que en *Polytomella* sp. el pH del medio de cultivo y la fuente de carbono tienen efectos en la acumulación del citocromo *c* y de los complejos respiratorios. Los espectros de las mitocondrias aisladas de las dos algas mostraron diferencias significativas en el contenido de citocromos. Además, se reportaron algunos resultados acerca de la regulación mitocondrial por la luz en *C. reinhardtii*. Una revisión de la literatura acerca de este tema en plantas y algas verdes describe que en la luz, las mitocondrias suelen dirigir su metabolismo hacia la disipación de reductores que se producen durante la actividad fotosintética, mientras que en la obscuridad lo dirigen esencialmente hacia la producción de ATP.

ABSTRACT

The genus Chlamydomonas is a member of the class Chlorophyceae, which contains more than 600 species, of which the vast majority is photosynthetic, although it also contains several colorless algae unable to perform photosynthesis. It was proposed that the colorless algae, such as those of the genus *Polytoma* and *Polytomella*, have lost an active photosynthetic apparatus. Selective environmental stress or interactions with other organisms, for example via lateral gene transfer, are possible causes of the loss of photosynthesis.

The green alga *Chlamydomonas reinhardtii* is extensively used as a model organism for photosynthesis. As the primary site of photosynthesis, the chloroplast of this alga has been well-studied. However, the mitochondria and their role in the metabolism of *C. reinhardtii*, and more generally in any photosynthetic cell, are poorly understood. Since light is one of the most important external factors for photosynthetic organisms, the effect of light on the mitochondrial function and biogenesis is interesting but has also received little attention.The heterotrophic *Polytomella* sp. constitutes a model organism for the study of the consequences of the loss of photosynthetic activity in the same metabolic framework as *C. reinhardtii*.

Polytomella sp. proved its close relationship with *C. reinhardtii* by the fact that it shares with this green alga several peculiarities in its respiratory complexes. However, major differences were also described that are probably caused by the loss of a functional photosynthetic apparatus. In this work, the major complexes of oxidative phosphorylation in *C. reinhardtii* and *Polytomella* sp. are described using isolated mitochondria and a proteomic approach. In both algae, the ATP synthase show an unusually strong dimeric behavior, and in addition, it exhibits several atypical subunits. The subunits of *C. reinhardtii* cytochrome *c* oxidase were found to possess several unusual features (like the presence of a fragmented COXII subunit). The surprising and unexplained presence of a bacterial enzyme involved in anaerobic metabolism, an aldehyde/alcohol dehydrogenase, was reported in the mitochondria of *Polytomella* sp., whereas a gene that encodes the same enzyme was identified in the genome of its photosynthetic relative *C. reinhardtii*.

In contrast to *C. reinhardtii*, *Polytomella* sp. can grow under a wide pH range and can use different carbon sources. It was shown that the pH and the carbon source influence the accumulation of the respiratory complexes and cytochrome *c*. In addition, spectra of isolated mitochondria showed differences in the cytochrome content between the two algae. Furthermore, some results on the light regulation of mitochondria are presented. A literature review on this theme in plants and green algae describes that in light, the mitochondrial metabolism is usually directed towards the dissipation of excess reductants resulting from photosynthetic activity, whereas in the dark, it is mainly directed to ATP production.

LISTA DE ABREVIATURAS

AcetilCoA	Acetil-coenzima A
ACS	Acetil-CoA sintasa
ADHE	Aldehído/alcohol deshidrogenasa bifuncional de tipo bacteriano
AOX	Oxidasa alterna
BN-PAGE	Electroforesis en geles azules nativos de poliacrilamida
CoA	Coenzima A
Complejo I	NADH:ubiquinona óxidorreductasa
Complejo II	Succinato:ubiquinona óxidorreductasa
Complejo III	Ubiquinona:citocromo *c* óxidorreductasa
Complejo IV	Citocromo *c* oxidasa
Complejo V	F_oF_1-ATP sintasa
CTAB	bromuro de N-cetil-trimetil-amonio
CTE	Cadena de transporte de electrones
Cyt	Citocromo
EST	'Expressed sequence tags'
FeS	Grupo fierro-azufre
GDC	Glicina descarboxilasa
H3	Medio de cultivo que contiene una alta concentración de acetato
ITS	Región traducida interna
NADH	Dinucleótido de nicotinamida adenina (reducida)
$FADH_2$	Dinucleótido de flavina adenina (reducida)
FMN	Mononucleótido de flavina
MPP	Peptidasa procesadora mitocondrial
OXPHOS	Fosforilación oxidativa
PDC	Complejo de la piruvato deshidrogenasa
PFL	Piruvato formato liasa
Q (CoQ)	Ubiquinona (coenzima Q)
QH_2	Ubiquinol (ubiquinona reducida)
redox	Reducción/oxidación
TAP	Medio de cultivo que contiene tris, acetato y fosfato.

El fin de la ciencia se acerca cuando nuevas respuestas dejan de causar nuevas preguntas... lo cual probablemente nunca sucederá. Estaremos a salvo.

INTRODUCCIÓN

INTRODUCCIÓN

La mitocondria: la fábrica de energía

La función principal de las mitocondrias en las células eucariontes es la producción de energía en forma de ATP a partir de glucosa y ácidos grasos. Sin mitocondrias, todo el ATP debería obtenerse de los procesos anaeróbicos, y no sería posible la vida de la mayoría de los organismos, incluyendo la humana. Además de la producción de ATP, otras funciones mitocondriales incluyen el mantenimiento del estado de reducción/oxidación (redox) en la célula, la detoxificación y la producción de calor (Zorov y cols., 1997). Las mitocondrias también tienen un papel importante en la apoptosis; es decir, la muerte celular programada (Kroemer y cols., 1995). La mitocondria está compuesta por dos membranas, el espacio intermembranal y la matriz (Figura 1). El plegamiento en crestas provee una superficie muy amplia a la membrana interna, aumentando así la capacidad de síntesis de ATP: la presencia de más crestas indica mayores requerimientos de energía.

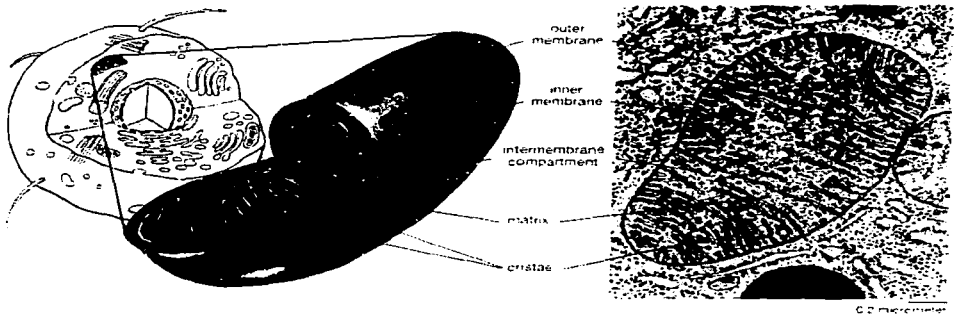

Figura 1. Estructura de la mitocondria e imágen de microscopia electrónica.

El ciclo Krebs es un conjunto cíclico de reacciones que se llevan a cabo en la matriz mitocondrial, que oxída metabolitos celulares generando CO_2, NADH, $FADH_2$ y ATP. La fosforilacón oxidativa es el proceso en el cual las mitocondrias producen grandes cantidades de ATP, utilizando los electrones provenientes del NADH, del $FADH_2$ y del oxígeno:

$$NADH + 3ADP + 3P_i + 4H^+ + O_2 \rightarrow NAD^+ + \underline{3ATP} + H_2O$$

Este proceso lo lleva a cabo un conjunto de componentes proteicos oligoméricos que se encuentran en la membrana interna, que forman la cadena respiratoria (Figura 2). Básicamente, la respiración es una cadena de transporte de electrones (CTE) que produce un gradiente de protones y un potencial electroquímico a través de la membrana interna. El gradiente electroquímico de protones es disipado por la ATP sintetasa (Figura 2), la cual produce ATP a partir de ADP y fosfato inorgánico (P_i). Esta teoría quimiosmótica, propuesta por Mitchell (1961, 1966), ha sido aceptada por toda la comunidad científica. La hipótesis quimiosmótica también explica la síntesis de ATP en las membranas de las bacterias y en los tilacoides de los cloroplastos de plantas (Gautheron, 1984).

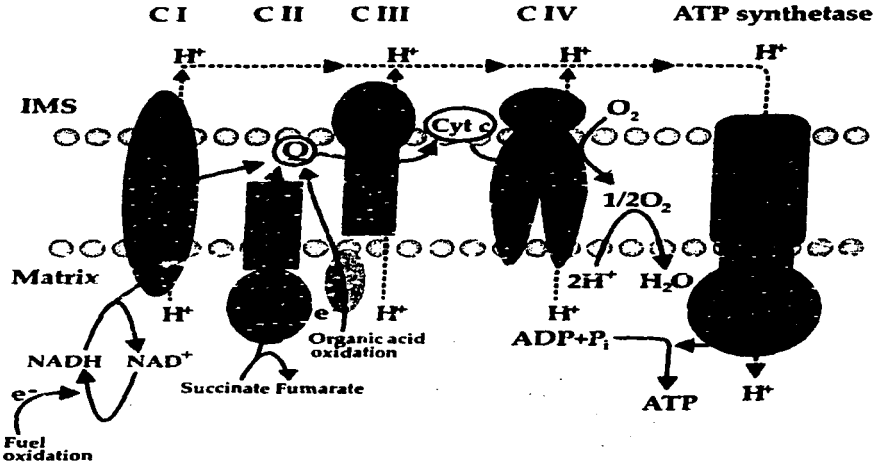

Figura 2. Los complejos de la cadena respiratoria mitochondrial. C I a C IV, complejo respiratorio I a IV, IMS, espacio intermembranal; Q, ubiquinona; Cyt *c*, citocromo *c*. La línea punteada indica el flujo de protones (H^+), las líneas continuas indica el flujo de electrones (e^-).

Aparte de la proteínas del ciclo Krebs y la CTE, se necesitan otras proteínas para la fosforilación oxidativa, Los transportadores o acarreadores transportan muchas moléculas a través de la membrana interna mitocondrial. Por ejemplo, el transportador de ADP/ATP transporta ADP hacia la matriz mitocondrial y al ATP hacia el citosol celular. También existen transportadores para P_i y piruvato (Palmieri, 1994).

Los componentes de la cadena respiratoria

Para comprender mejor la estructura y función de la cadena respiratoria mitocondrial, se describe a continuación con más detalle cada uno de sus componentes. Se toma como ejemplo la cadena respiratoria de los mamíferos, la cual es la que mejor se ha caracterizado.

Complejo I

NADH:ubiquinona óxidorreductasa. Este complejo es el componente proteico más grande de la cadena y está compuesto por 45 subunidades (Carroll y cols., 2002); tiene la forma de una 'L', constituida por una parte membranal y una parte periférica que es hidrofílica. El brazo periférico de la L, que destaca hacia la matriz, es el sitio de la oxidación del NADH. Los electrones del NADH son enviados hasta la ubiquinona por varias subunidades que contienen un mononucleótido de flavina (FMN), y varios centros fierro-azufre (FeS). En este complejo, por cada dos electrones, se translocan cuatro protones a través de la membrana interna mitocondrial (Brandt, 1997).

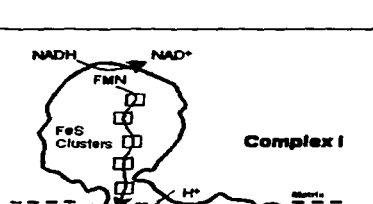

Figura 3. Estructura del complejo I. Los grupos *redox*, involucrados en el transporte de los electrones están localizados en el brazo soluble. La forma del complejo es según Grigorieff (1998).

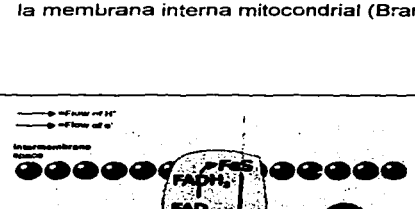

Figura 4. Flujo de electrones por el complejo II a la CoQ. Nótese que este complejo no bombea electrones. La interacción de la CoQ con el complejo III produce una translocación de protones.

Complejo II

Succinato:ubiquinona óxidorreductasa. Este complejo enzimático es también parte del ciclo Krebs, siendo la única enzima de este ciclo asociada a la membrana interna mitocondrial. Con tres o cuatro subunidades, el complejo II es el más pequeño de la cadena respiratoria. La función del complejo II es donar electrones del ciclo Krebs a la ubiquinona (Hägerhäll, 1997). Este complejo no bombea protones.

Ubiquinona

La ubiquinona es una molécula muy hidrofóbica que está presente en la membrana interna, y que recibe electrones del complejo I o II y los transfiere al complejo III. La ubiquinona no solo funciona como un transportador de electrones en la cadena respiratoria, sino también como un antioxidante en muchas membranas celulares (Pobezhimova y Voinikov, 2000).

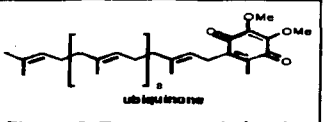

Figura 5. Estructura química de la ubiquinona. Los átomos de oxígeno son los sitios de la reducción (O-H) y oxidación (=O). Me=grupo metileno (CH_3).

Complejo III

Ubiquinona:citocromo *c* óxidorreductasa. Esta enzima está compuesta por 11 subunidades, y oxida la ubiquinona para reducir al citocromo *c*. Esta reacción está acoplada al movimiento de cuatro protones a través de la membrana interna, mediante el llamado ciclo Q. El citocromo *b* del complejo III contiene dos grupos hemo que tienen distintos potenciales redox (b_{560} y b_{566}). En cada ciclo, de los dos electrones del ubiquinol, un electrón es transportado por la proteína Rieske (FeS) al citocromo c_1 para reducir el citocromo *c*; mientras que el otro electrón es transportado por los dos hemos *b* para formar ubisemiquinona (Q^-) a partir de ubiquinona en el otro lado de la membrana interna. La repetición de este ciclo Q produce otro ubiquinol, y resulta en el transporte neto de cuatro protones por la membrana interna (Trumpower, 1990). El complejo III también contiene dos proteínas estructurales (core proteins), que son homólogas a las peptidasas mitocondriales (MPP), involucradas en la maduración de las preproteínas importadas desde el citoplasma. En las plantas, se ha establecido que las proteínas estructurales efectivamente tienen función de MPP (Braun y Schmitz, 1995a).

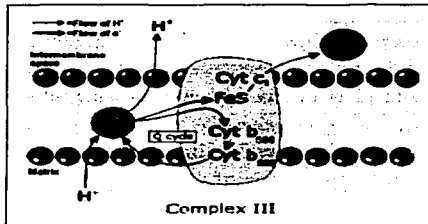

Figura 6. Flujo de electrones por el complejo III de la ubiquinona al citocromo *c*. Los dos electrones de la CoQ están separados, uno pasa directamente al citocromo *c*, mientras el otro pasa por el ciclo Q, bombeando protones.

Citocromo *c*

Es una proteína soluble de 12 kDa, que contiene un grupo hemo de tipo *c* como grupo redox, ligado covalentemente por dos residuos de cisteína. El citocromo *c* transporta los electrones del complejo III al complejo IV y está situado en el espacio intermembranal (Pettigrew y Moore, 1987).

Figura 7. Estructura cristalográfica del citocromo *c*. El grupo hemo tipo *c* está presente en el centro.

Complejo IV

Citocromo *c* oxidasa. Es el componente terminal de la cadena de transporte de electrones. Este complejo enzimático contiene 13 subunidades. Tres subunidades constituyen el centro de la proteína, numeradas I, II y III. La subunidad I contiene el sitio activo, con dos hemos tipo A y un átomo de cobre (Cu_A). La subunidad II tiene un centro de dos átomos de cobre (Cu_B), y recibe los electrones del citocromo *c* soluble. Los electrones son transportados al citocromo *a* y luego al centro binuclear formado por un cobre Cu_b y un hemo, el sitio de la reducción de oxígeno. La función de la subunidad III no ha sido aclarada. El transporte de electrones causa la translocación de cuatro protones por la membrana interna, sin embargo todavía no se ha establecido el mecanismo preciso de la translocación (Saraste, 1999). La citocromo *c* oxidasa es responsable de más del 90% del consumo de oxígeno de todos los organismos en el biósfera.

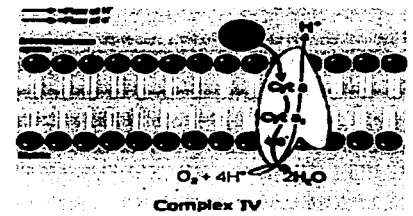

Figura 8. El transporte de electrones en el complejo IV, del citocromo *c* al oxígeno para formar agua.

Complejo V

F_oF_1-ATPsintasa. Este complejo tiene una masa molecular de más de 500 kDa, y está constituído por 16 subunidades distintas. La fuerza protón-motríz generada por los complejos I, III y IV es utilizada por la ATP sintasa para producir ATP. Por cada ATP sintetizado, se translocan tres o cuatro protones (Boyer, 1997). La sección membranal (F_o) contiene un canal para los protones, y está conectada con la parte catalítica soluble (F_1) que está expuesta hacia la matriz. La F_o está compuesta por lo menos de 9 subunidades, de las cuales sólo las subunidades *a*, *b* y *c* (estoquiometría 1:1:12) tienen hornólogos en *E. coli*. La F_1 (371 kDa en bovino) está compuesta por 5 subunidades α, β, γ, δ y ε en una estoquiometría 3:3:1:1:1. La subunidad γ interactúa con las subunidades ε y *c* (ATP9) para formar la parte conocida como el rotor, mientras que la parte llamada estabilizador está compuesta por las subunidades α, β, δ, *a* (ATP6) y *b*, formando un segundo lazo entre la F_o

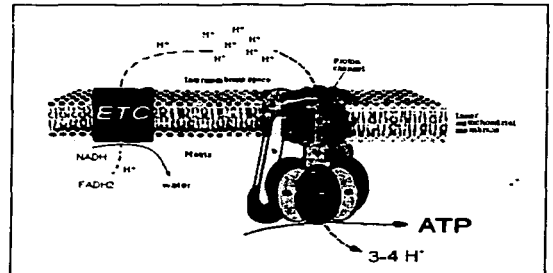

Figura 9. Estructura de la F_oF_1-ATPsintasa. La oxidación de reductores por la CTE resulta finalmente en la producción de ATP.

y la F_1. La subunidad β posee el sitio catalítico, por lo que la F_1 tiene tres sitios catalíticos. El paso de los protones por la F_0 causa el giro del rotor, que genera tres distintos estados conformacionales: un estado sin sustrato (descargado de ATP), un estado con ADP y P_i enlazado, y un estado con ATP enlazado. La rotación impone la transición entre estos estados. La subunidad γ funciona como el eje de rotación de la F_1. La ATP sintasa también puede funcionar como ATPasa, en este caso la hidrólisis de ATP provee la energía para translocar protones y generar un gradiente electroquímico (Abrahams y cols., 1994; Pedersen y cols., 2000).

La mitocondria en las células fotosintéticas

En la célula fotosintética, la presencia de dos organelos que producen ATP, el cloroplasto y la mitocondria, trae complicaciones en el metabolismo redox. En la luz, la CTE del cloroplasto, que tiene mucho en común con la de la mitocondria, transporta electrones y lleva a cabo la producción de ATP (fotofosforilación) con principios semejantes a lo que ocurre en la mitocondria:

$$2H_2O + 2\ NADP^+ + nADP + nP_i + h\nu \rightarrow O_2 + 2NADPH + 2H^+ + \underline{n}ATP$$

Sin embargo, aún cuando el cloroplasto funcione en presencia de luz, la mitocondria sigue produciendo ATP para procesos como la síntesis de sacáridos y la fijación de nitrógeno. En la obscuridad, la mitocondria es el único sitio de la síntesis de ATP.

La mitocondria es necesaria para optimizar el metabolismo fotosintético bajo una variedad de condiciones ambientales (Krömer 1995; Gardeström y Lernmark 1995; Hoefnagel y cols., 1998; Padmasree y cols., 2002). La fosforilación oxidativa parece balancear el estado celular redox y energético. Por ejemplo, la inhibición selectiva de la ATPasa mitocondrial por la oligomicina da lugar a una inhibición parcial de la fotosíntesis en las hojas de chícharo illuminadas (Krömer y cols., 1988). También existe evidencia de que la función mitocondrial disipa el exceso de reductores producidos en el cloroplasto (Raghavendra y cols., 1994; Krömer 1995; Padmasree y Raghavendra 1999; Igamberdiev y cols., 2001a, b).

Para los organismos fotosintéticos, la luz es una fuente de energía pero también constituye una fuente de información sobre el ambiente. El estado de reducción en el cloroplasto aumenta drásticamente con la exposición a la luz (Scheibe, 1991), la cual también tiene un papel importante en la regulación de la expresión de genes y de la actividad de varias enzimas (Danon y Mayfield 1994; Allen y cols., 1995b; Pfannschmidt y cols., 1999a y 1999b). La luz también tiene efectos sobre la expresión de genes y sobre la actividad enzimática de varias proteínas

mitocondriales. Los mecanismos involucrados en esta regulación no se conocen, pero una posibilidad es que la mitocondria responda a mensajes redox provenientes del cloroplasto (artículo VI).

La cadena respiratoria de las mitocondrias de las células fotosintéticas

La Figura 10 muestra una imagen esquemática de la CTE mitocondrial de una célula fotosintética como *C. reinhardtii*. Comparadas con las CTE de mamíferos, las CTE en las mitocondrias de las plantas y de las algas verdes poseen componentes proteicos adicionales.

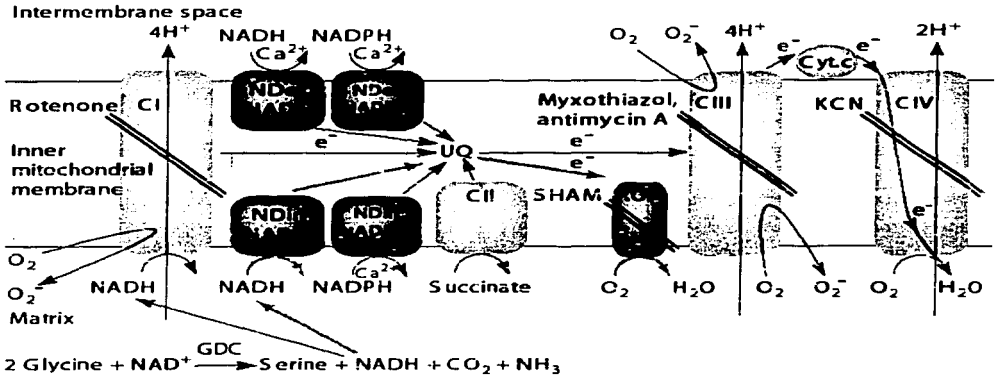

Figura 10. Representación esquemática de la cadena respiratoria mitochondrial de *C. reinhardtii* y otros organismos fotosintéticos (tomado de Møller, 2002). CI – CIV, complejos respiratorios I – IV; UQ, ubiquinona; cyt. c, citocromo *c*. Los componentes adicionales típicos de la cadena respiratoria de los organismos fotosintéticos se muestran en gris obscuro. GDC, glicina descarboxilasa; NDin/NDex, NAD(P)H deshidrogenasa insensible a rotenona, interior y exterior, respectivamente, y AOX, oxidasa alterna. Las líneas negras dobles indican la inhibición del complejo por la sustancia indicada. KCN, cianuro de potasio, SHAM, ácido salicil-hidroxámico.

NAD(P)H deshidrogenasas alternas (NDin/NDex)

Las cuatro NADH deshidrogenasas insensibles a rotenona presentes en las mitocondrias de las plantas forman parte de una ruta respiratoria no-fosforilante. Dos de estas enzimas están presentes en la superficie de la membrana interna expuestas hacia el espacio intermembranal (externas, NDex), y las otras dos están expuestas hacia a la matriz mitocondrial (internas, NDin).

Junto con la AOX, estas NAD(P)H deshidrogenasas permiten la respiración sin generación de gradiente electroquímico y sin producción de ATP. Las enzimas externas pueden utilizar reductores provenientes del citoplasma, mientras que las enzimas internas pueden oxidar al NAD(P)H producido en la matríz mitocondrial (Møller, 2002).

Oxidasa alterna (AOX)

La enzima se encuentra principalmente en plantas, protistas (como las algas verdes) y algunos hongos (Umbach y Siedow, 2000). La AOX es una oxidasa terminal (Figura 11), que desvía a la CTE ortodoxa a nivel de la ubiquinona. La AOX es una proteína homodimérica con monómeros de 36 kDa, que pueden estar enlazados covalentemente por un puente disulfuro (Figura 11). La forma oxidada es menos activa que la forma reducida, una característica que está relacionada con la regulación de la actividad de esta enzima. El monómero está codificado por el gen nuclear *Aox*1. Ya que la AOX no transloca protones, no participa en la producción de ATP. Se piensa que su papel principal es la disipación del exceso de reductores (Vanlerberghe y McIntosh, 1997).

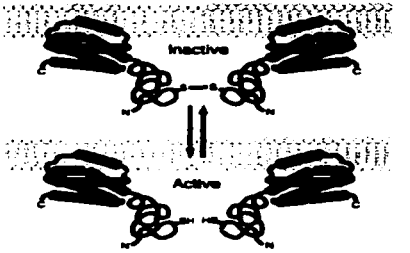

Figura 11. Interconversión entre el estado oxidado y reducido del dímero de la AOX

La oxidación del NAD(P)H y del succinato por la CTE mitocondrial es importante para optimizar la fotosíntesis. El exceso de reductores que se origina por la fotosíntesis puede ser exportado del cloroplasto y ser utilizado por la CTE mitocondrial (Hoefnagel y cols., 1998; Mackenzie y McIntosh, 1999; Padmasree y cols., 2002). Los componentes adicionales intrínsecos de las CTEs de plantas no bombean protones, así que estos componentes meramente funcionan como una poza redox (del inglés "redox sink"). De esta manera, la cadena respiratoria mitocondrial puede balancear el estado redox celular (NAD(P)H, $FADH_2$) y el estado energético (ADP/ATP) usando adecuadamente los componentes clásicos y los caminos alternativos no-fosforilantes. En el artículo VI se analiza en detalle el conocimiento actual acerca del papel de las mitocondrias en la fotosíntesis y la manera como la fotosíntesis regula la expresión de proteínas mitocondriales.

El complejo de glicina descarboxilasa (GDC), en combinación con la serina hidroximetil transferasa (SHMT), se encuentran en muchos organismos. Estas enzimas convierten glicina en serina (Douce y cols., 2001). En los organismos fotosintéticos, las enzimas están localizadas en

la matriz mitocondrial y están relacionadas con la fotosíntesis (Mackenzie y McIntosh, 1999). En la luz, y bajo condiciones de CO_2 limitantes para la fijación de carbono, esta enzima puede producir grandes cantidades de NADH, lo cual estimula la respiración. En estas condiciones, las enzimas respiratorias que no bombean protones (el camino AOX) están activadas (Igamberdiev y cols., 1997; Svensson y Rasmusson, 2001), con el fin de evitar la sobrerreducción de la CTE y para balancear la demanda de ATP y el metabolismo de carbono en el ciclo Krebs (Vanlerberghe y McIntosh, 1997).

Aislamiento y caracterización de las mitocondrias

El primer paso para estudiar los procesos mitocondriales es el aislamiento de las mitocondrias de los tejidos o células del organismo de interés. El método básico de todos los protocolos para aislar mitocondrias es el mismo y consiste en la ruptura del tejido y las células, seguida de centrifugaciones diferenciales. Pueden requerirse pasos adicionales, como una centrifugación en gradiente de densidad, para eliminar restos de material contaminante, como las membranas tilacoides de plantas o algas verdes (Neuburger y cols., 1982). Las mitocondrias pueden ser estudiadas y caracterizadas por diferentes tipos de análisis, como la medición del consumo de oxígeno, la importación de proteínas, el aislamiento del DNA mitocondrial, la espectrofotometría y la electroforesis en geles de poliacrilamida (Figura 12).

Para medir el consumo de oxígeno, se colocan las mitocondrias en una celda con un electrodo de Clark. La adición de sustratos para la respiración, como succinato o malato, inicia el consumo de oxígeno; el uso de inhibidores de los distintos componentes de la CTE puede dar información sobre la función y capacidad de la respiración mitocondrial. Estos estudios también se pueden llevar a cabo con células enteras, para investigar el papel de las mitocondrias en el metabolismo celular.

Para caracterizar los componentes de la CTE, en particular los citocromos presentes en los complejos III y IV y el citocromo *c*, se pueden registrar los espectros de las mitocondrias o de las membranas mitocondriales. La electroforesis en geles de poliacrilamida (PAGE) también es un método poderoso para estudiar la biosíntesis mitocondrial. Aparte de la clásica SDS-PAGE, existen métodos más versátiles de PAGE que permiten analizar el proteoma mitocondrial en su conjunto.

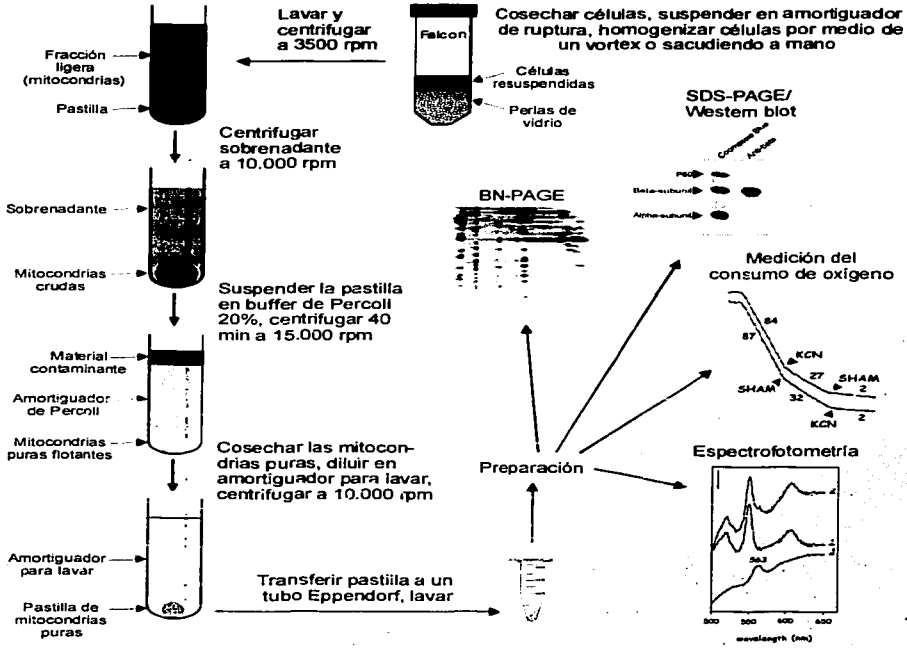

Figura 12. Protocolo para el aislamiento de mitocondrias de *C. reinhardtii* y los posibles análisis que se pueden hacer a las mitocondrias puras. Para la medición del consumo de oxígeno, se pueden utilizar mitocondrias crudas. Ya que *Polytomella* sp. no tiene cloroplastos, para el aislamiento de mitocondrias de esta alga incolora, se omite el gradiente de Percoll.

La proteómica: electroforesis nativa en geles azules (BN-PAGE)

Una herramienta para estudiar los complejos respiratorios mitocondriales es la electroforesis en geles de poliacrilamida en dos dimensiones (2D-PAGE). Se ha usado mucho la electroforesis de isoelectroenfoque (IEF), para separar proteínas de acuerdo con su punto isoeléctrico (pI), aplicando un gradiente de pH en el gel. Sin embargo, la aplicación del IEF a

proteínas membranales es difícil, ya que se pierden las interacciones de las subunidades de las proteínas oligoméricas. Para superar estos problemas, se ha usado electroforesis en geles nativos azules de poliacrilamida (BN-PAGE). Pueden solubilizarse membranas u organelos enteros con detergentes no-iónicos (como lauril-maltósido o Tritón X-100), que no destruyen las interacciones de las subunidades. Posteriormente, la adición de azul de Coomassie a la muestra y su unión a las proteínas, genera una carga negativa que le da movilidad a las proteínas en un campo electroforético (Schägger y von Jagow, 1991). De esta manera, se conserva la integridad de los complejos proteicos, pero se promueve su separación por tamaño en un campo electroforético. Los complejos separados en geles azules (primera dimensión) pueden ser desnaturalizados con la adición de SDS y mercaptoetanol, para después ser analizados en geles SDS-PAGE (segunda dimensión) para conocer su composición polipeptídica. Se han analizado los complejos respiratorios de varios organismos utilizando BN-PAGE, entre otros los complejos de bovino (Schägger y von Jagow, 1991), de papa, de *Arabidopsis* (Jänsch y cols., 1996), de levadura (Schägger y Pfeiffer, 2000), y de las algas unicelulares *Chlamydomonas reinhardtii* (artículo I) y *Polytomella* sp. (artículo V). Como se muestra en la Figura 13, los complejos más abundantes de las mitocondrias de papa fueron separados en un gel nativo azul, y después desnaturalizados en 2D-SDS-PAGE. Las bandas de proteína se identificaron por medio de su secuencia de aminoácidos por degradación de Edman, o por espectometría de masas. Estas aplicaciones hacen del BN-PAGE un buen método para llevar a cabo un enfoque proteómico, y permiten la descripción parcial de los proteomas de fracciones celulares (como mitocondrias y cloroplastos) de diferentes tipos de organismos.

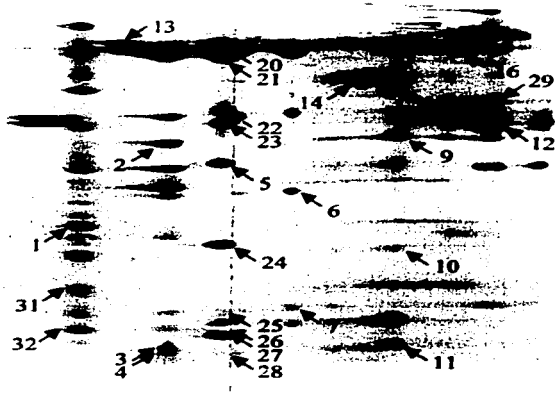

Figura 13. Gel desnaturalizante de dos dimensiones de BN-PAGE. Los complejos proteicos mayores de papa (tomado de Jänsch y cols., 1996). Números: **1,31,32**: subunidades de 18, 12 y 8 kDa del complejo I; **2,4,3**: subunidades de 27 y 6 kDa y subunidad ATP9 del complejo V (F_O); **20,21,22,23,5,24,25,26,27,28**: subunidades del complejo III, beta-MPP, alfa-MPP, citocromo *b*, citocromo c_1, Rieske, 14 kDa, Hinge, 8.2 kDa, 8.0 kDa, 6.7 kDa; **6,7**: subunidad delta e inhibidora del complejo V (F_1); **9,10,11**: subunidades coxII, coxVb y coxVc del complejo IV; **13**: HSP60; **14**: formato deshidrogenasa; **30**: porina; **16**: glicina descarboxilasa, subunidad L; **29**: NAD-malato deshidrogenasa; **12**: porina.

Regulación de la respiración mitocondrial y la fosforilación oxidativa

En los mamíferos, la respiración mitocondrial está regulada por distintos factores, que incluyen el estado redox y el estado energético en la celúla. Las relaciones ATP/ADP y NADH/NAD, así como el tipo de sustratos que sostienen la respiración son muy importantes en la regulación mitocondrial (Brown, 1992). La disponibilidad de ATP y ADP tiene un efecto importante en la función mitocondrial, ya que los niveles de estas moléculas representan la demanda energética de la célula. Por ejemplo, se ha reportado que el ADP inhibe al complejo V, disminuyendo la producción de ATP (Martins y cols., 1988). Además, el ATP inhibe alostéricamente al complejo IV al unirse a la subunidad IV. Esto ocurre en eucariontes, pero no así en bacterias (Arnold y Kadenbach, 1997; Follmann y cols., 1998). Los cambios en el tipo y concentración de los sustratos respriratorios, como el piruvato y los ácidos grasos, también tienen un efecto notable sobre la respiración, cambiando la relación NADH/NAD en la mitocondria. Un aumento en el nivel de NADH causa un aumento del transporte de electrones por la CTE y de la síntesis de ATP (Brown, 1992; Wilson, 1994). El calcio también puede funcionar como mensajero para ajustar los niveles de NADH en la mitocondria, cuando un incremento en la demanda de ATP en el citoplasma (de células musculares) causa un aumento de la cantidad de calcio. El transporte del calcio a la mitocondria activa las deshidrogenasas que producen NADH, lo cual a su vez estimula la fosforilación oxidativa (McMillin y Madden, 1989).

En las mitocondrias de plantas y algas verdes, los mecanismos de regulación mencionados anteriormente se aplican sólo hasta cierto punto, ya que la regulación de la función mitocondrial se complica por la presencia del cloroplasto, que produce también ATP, reductores y sustratos. Los organismos fotosintéticos dependen de la luz; en especial las algas unicelulares están en contacto directo con el ambiente y no pueden controlar la temperatura de sus células, por lo que el metabolismo celular está profundamente influenciado por los factores ambientales, como la luz, la temperatura, los metales, los nutrientes, y el pH. A pesar del interés que representa estudiar los mecanismos de regulación en las algas unicelulares, las mitocondrias de plantas y algas verdes han sido poco estudiadas debido a la dificultad que representa aislar mitocondrias libres de contaminación de membranas/proteínas del cloroplasto. Se ha reportado ejemplos de regulación por la luz de la función y la biogénesis mitocondrial en organismos fotosintéticos, mientras se conocen algunos efectos de la temperatura en las mitocondrias. La adaptación mitocondrial a bajas temperaturas involucra la activación de la AOX para generar calor (Atkin y cols., 2002), mientras que la respuesta a las condiciones de luz también involucra a los componentes adicionales mitocondriales, mencionados en la Figura 10. La AOX, las NADH deshidrogenasas alternas y la GDC se inducen en la luz (Walker y Oliver, 1986; Finnegan y cols., 1997; Svensson y Rasmusson, 2001), lo cual indica la participación de esas enzimas en el metabolismo fotosintético. También otros componentes mitocondriales están regulados por luz

(Landschutze y cols., 1995; Long y Berry, 1996). En el artículo VI se analiza con más detalle la regulación mitocondrial por la luz en plantas y algas verdes.

El alga verde *Chlamydomonas reinhardtii*

Aunque los protistas unicelulares parecen ser organismos eucariontes muy simples, a nivel celular presentan una gran complejidad. Una sola célula debe cumplir con todas las funciones básicas, las cuales son llevadas a cabo por varias células o tejidos especializados en plantas y animales.

Chlamydomonas reinhardtii es un alga unicelular biflagelada que es capaz de realizar fotosíntesis (Figura 14). Ya que esta alga realiza todas las funciones necesarias en una célula, es un modelo biológico excelente para estudiar varios fenómenos. De hecho, *C. reinhardtii* fue adoptada como modelo para estudiar la fotosíntesis y por eso se le ha llamado 'la levadura fotosintética' (Rochaix, 1995). Hasta la fecha, la gran mayoría de los estudios realizados con *C. reinhardtii* se han dedicado al ensamblaje flagelar y a la biogénesis y función del cloroplasto en el alga (Rochaix, 1995; Harris, 2001).

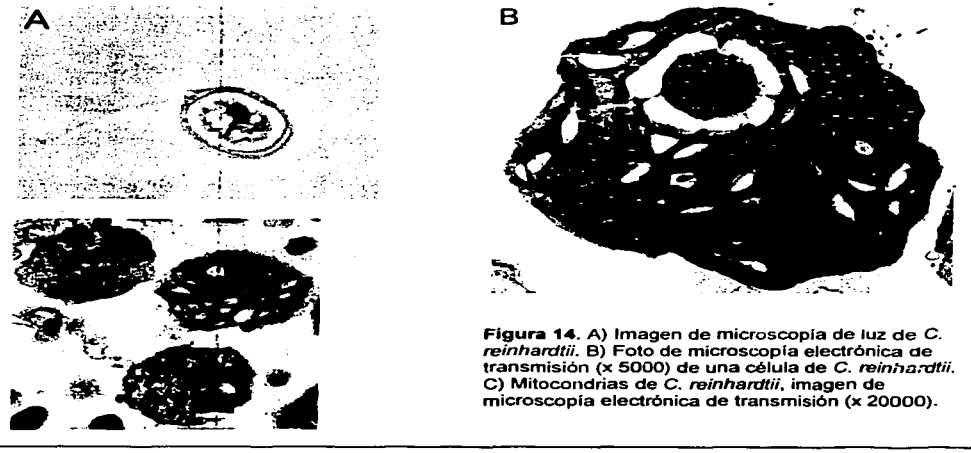

Figura 14. A) Imagen de microscopía de luz de *C. reinhardtii*. B) Foto de microscopía electrónica de transmisión (x 5000) de una célula de *C. reinhardtii*. C) Mitocondrias de *C. reinhardtii*, imagen de microscopía electrónica de transmisión (x 20000).

C. reinhardtii se considera como un organismo muy útil para estudiar procesos fotosintéticos y procesos relacionados por sus herramientas genéticas y bioquímicas, que son muy versátiles; sin embargo, se han hecho relativamente pocos estudios sobre sus mitocondrias. Esto se debe a la dificultad de obtener mitocondrias puras en cantidades suficientes. El método desarrollado por Ericksson y cols. (1995) permitió el aislamiento de mitocondrias de esta alga, esencialmente libres de contaminación por membranas tilacoidales (Figura 14). A pesar de la disponibilidad de este protocolo, la mayoría de los grupos de investigación han optado por trabajar con mitocondrias de organismos de los cuales se puede obtener material con más facilidad, como el tubérculo de la papa. La pregunta es, ya que el tejido mismo del tubérculo de la papa no es fotosintético, hasta que punto las mitocondrias de papa son representativas de los tejidos fotosintéticos. Las mitocondrias de los organismos fotosintéticos son de interés, ya que estos organelos no sólo producen energía, si no también tienen un papel importante en la optimización del proceso de la fotosíntesis (Padmasree y cols., 2002). Como ya se mencionó, esto se refleja por la presencia de varios componentes proteicos adicionales en las mitocondrias de plantas y algas, comparado con, por ejemplo, las mitocondrias animales. La mayoría de estos componentes adicionales se encuentran como parte de la cadena respiratoria o directamente asociados con ella. Es probable que la investigación de las mitocondrias de organismos o tejidos fotosintéticos contribuya a una mejor comprensión de la fotosíntesis, la cual después de todo es la base de toda la vida.

Otra razón por la que es interesante estudiar las mitocondrias de *C. reinhardtii*, es el carácter original de su genoma y de su proteoma mitocondrial. En comparación con las plantas, el genoma mitocondrial del alga es mucho más pequeño (15.8 kbp) y no es circular, sino lineal (Michaelis y cols., 1990). Además, este DNA mitocondrial carece de varios genes que normalmente se encuentran en los genomas mitocondriales, como *nad*3 (complejo I), *cox*2, *cox*3, (complejo IV), *atp*6 y *atp*8 (complejo V).

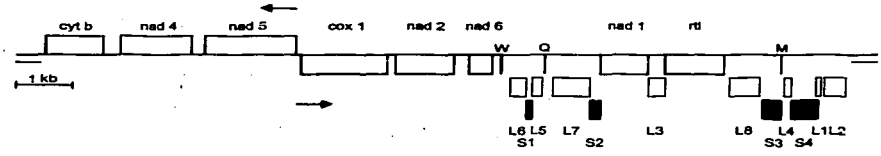

Figura 15. El genoma mitocondrial lineal de *C. reinhardtii* (15.8 kbp). En blanco, los genes que codifican para proteínas; en gris y en negro los genes que codifican para los rRNAs grandes y pequeños, respectivamente; W, Q y M indican los genes de los tRNAs para los aminoácidos corespondientes (Figura de Michaelis y cols., 1990)

Se ha demostrado que los genes que codifican para las subunidades II y III de la citocromo oxidasa en *C. reinhardtii* han sido transferidos al genoma nuclear (Pérez-Martínez y cols., 2000, 2001). La subunidad COXII está fragmentada en dos porciones polipeptídicas COXIIa, y COXIIb, codificadas por los genes nucleares *cox2a* y *cox2b* respectivamente (Pérez-Martínez y cols., 2001). En el artículo I, se identificaron los productos proteicos codificados por los genes *cox2a*, *cox2b* y *cox3*. También, se ha clonado el gen nuclear *atp6* que codifica a la subunidad ATP6 en *C. reinhardtii* y también se ha identificado su producto proteico (Artículo III). Se ha demostrado que la transferencia de los genes *cox2*, *cox3* y *atp6* al genoma nuclear se acompañó por la adquisición de una secuencia que codifica para una presecuencia mitocondrial, necesaria para la importación de estas proteínas a la mitocondria. Las subunidades COXIIa, COXIIb, COXIII y ATP6 presentan una hidrofobicidad disminuída, lo cual podría facilitar la importación de estas proteínas a la mitocondria (Pérez-Martínez y cols., 2000, 2001; Artículo III).

Las subunidades α y β de la F_1-ATP sintasa en el alga verde tienen extensiones atípicas en sus extremos amino terminal y carboxilo terminal, respectivamente (Fränzen y Falk, 1992; Nurani y Fränzen, 1996). No se ha investigado el papel functional de estas extensiones.

La familia no-fotosintética de *C. reinhardtii*: *Polytomella* sp.

El otro organismo que se ha usado para estudios de las mitocondrias es el alga cuadriflagelar no fotosintética *Polytomella* sp. (Figura 16), un pariente cercano de *C. reinhardtii*. El aislamiento de las mitocondrias es mucho más fácil, porque *Polytomella* sp. carece de un aparato fotosintético funcional y de una pared celular. Los remanentes de un cloroplasto ancestral se manifiestan en forma de amiloplastos. A pesar de las diferencias, las mitocondrias de esta alga incolora tienen aspectos muy similares a las de su homólogo verde *C. reinhardtii* (véase más adelante). *Polytomella* sp. ha demostrado ser un organismo muy útil para la descripción de los componentes de la cadena respiratoria de *C. reinhardtii* y probablemente mostrará su utilidad para estudiar la adaptación de las algas a las condiciones ambientales (Artículo II). Considerando que *Polytomella* sp. carece de cloroplastos funcionales, puede también servir para estudiar el papel de las mitocondrias en la fotosíntesis. La comparación de la función y biosíntesis de las mitocondrias de las dos algas podría revelar nuevos aspectos del metabolismo de las células fotosintéticas.

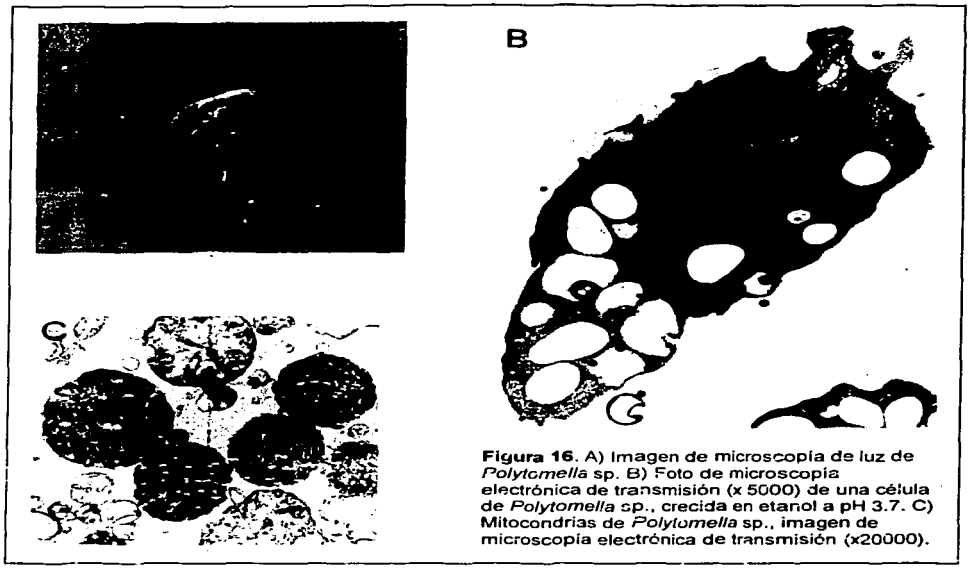

Figura 16. A) Imagen de microscopía de luz de *Polytomella* sp. B) Foto de microscopía electrónica de transmisión (x 5000) de una célula de *Polytomella* sp., crecida en etanol a pH 3.7. C) Mitocondrias de *Polytomella* sp., imagen de microscopía electrónica de transmisión (x20000).

Comparación filogenética y bioquímica de *C. reinhardtii* y *Polytomella* (sp.)

Las algas de los géneros *Chlamydomonas* y *Polytomella* son parte del phylum de las Chlorophyta, la clase de las Chlorophyceae, del orden de las Chlamydomonadales, familia de Chlamydomonadaceae (Melkonian, 1990). Sin embargo, recientemente el análisis de de las secuencias del 18S rRNA de las algas clorofíceas ha resultado en una clasificación distinta de estas algas, detallado abajo (Pröschold y cols., 2001). Las algas clorofíceas están ubicadas dentro del lineaje verde. Este grupo incluye todas las algas verdes (algunas incoloras) que a su vez son parientes más lejanos de las plantas (Figura 17). Los eucariontes están divididos en varios grupos que surgieron en relativamente poco tiempo (desde el punto de vista evolutivo). Los primeros eucariontes fotosintéticos se encontraban presentes antes de la radiación ('crown group radiation', Figura 17) en una gran cantidad de organismos diversos.

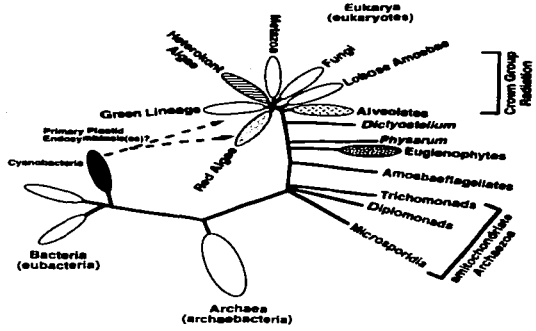

Figura 17. La filogenia de los tres dominios de la vida, basada en la comparación de las secuencias de la subunidad pequeña del rRNA (Tomado de Bhattacharya y Medlin, 1998).

Las algas clorofíceas se pueden dividir en distintos grupos (clados), según las secuencias de la subunidad pequeña del RNA ribosomal nuclear (18S) o de la región traducida interna (ITS). Pröschold y cols. (2001) reportaron un análisis filogenético muy extenso del género *Chlamydomonas* basado en la comparación de las secuencias del rRNA 18S. En este análisis, *Polytomella parva* se encuentra como grupo cercano al grupo de *C. reinhardtii*. Tomando la secuencia del ITS de *Polytomella* sp. (SAG 198.80), el alga está aún más cercana a *C. reinhardtii* que a *Polytomella parva* (comunicación personal T. Pröschold). Se supone que la ITS es un marcador que puede distinguir mejor que la 18S a nivel de las especies. Estos datos indican la gran cercanía entre *C. reinhardtii* y *Polytomella* sp. a nivel del rRNA.

Las dos algas son homólogas no sólo a nivel de las secuencias del rRNA nuclear; también muestran muchas similitudes entre sus genomas mitocondriales, así como muchas semejanzas bioquímicas entre sus proteínas mitocondriales. El tamaño y el contenido de genes del genoma mitocondrial de *Polytomella parva* parece ser similar al genoma de *C. reinhardtii*, aparte del hecho que el gen *nad6* (complejo I) está codificado por un fragmento separado de DNA de 3.5 kbp (Fan y Lee, 2002). En *Polytomella* sp. también existe un fragmento pequeño de DNA de 3.5 kbp (observación personal). Una indicación fuerte de la similitud de *C. reinhardtii* y *Polytomella* se encuentra en la subunidad COXII del complejo IV: como en el alga verde, también en *Polytomella* sp., esta proteína está codificada por dos genes nucleares, cox2a y cox2b (Pérez-Martínez y cols., 2001).

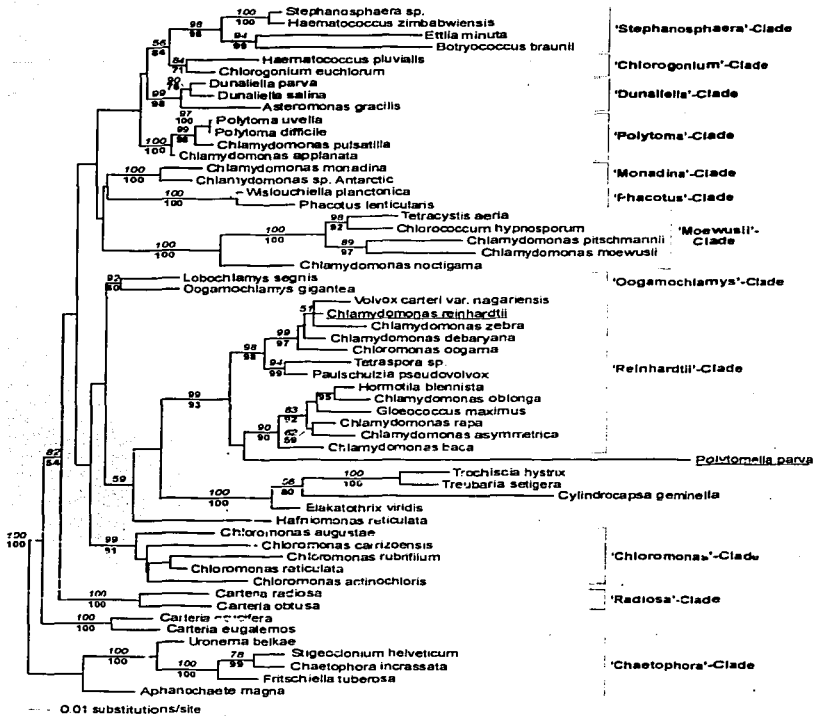

Figura 18. Filogenia molecular del grupo 'CW' (*sensu* Pröschold y cols., 2001; Clorofíceas), que contiene *Chlamydomonas* y *Polytomella* (subrayado; notar que *Polytomella parva* no es igual que *Polytomella* sp.), basado en las comparaciones de las secuencias del 18S rRNA, codificado por un gen nuclear. El árbol filogenético se dedujo del método de máxima verosimilitud y calculado con el modelo de Tamura y Nei (TrN+I+G; Tamura y Nei, 1993), usando 1717 posiciones alineadas. Los números indican los valores de bootstrap (>50%) para cada rama (cursivas, método del vecino más cercano; negritas, método de la máxima parsimonia no sopesada). El árbol es una contribución de T. Pröschold (Universidad de Colonia, Alemania).

En la sección de los resultados adicionales se encuentran algunos resultados de estudios sobre las mitocondrias de las dos algas, que revelan que hay algunas diferencias importantes, en particuiar en el contenido de citocromos.

ANTECEDENTES

Los complejos respiratorios de *C. reinhardtii* fueron parcialmente caracterizados por SDS-PAGE y espectrofotometría (Atteia y cols., 1992; Atteia, 1994). Después se aisló el complejo V y se caracterizaron las subunidades alfa y beta (Nurani y Franzén, 1996). Hasta la fecha, no se había llevado a cabo una caracterización profunda de los otros complejos respiratorios, debido a la dificultad de aislar mitocondrias puras del alga verde. Hace algunos años, se describió un protocolo para aislar mitocondrias de *C. reinhardtii* libres de contaminantes del cloroplasto (Eriksson y cols., 1995), lo cual abrió el camino a estudios más detallados de la biogénesis y la regulación de las mitocondrias.

El estudio de la regulación mitocondrial en relación con la fotosíntesis es de gran interés, especialmente en diferentes condiciones de luz, ya que la luz es la fuente de energía de la fotosíntesis, pero también una fuente de información sobre el ambiente. Tanto en *C. reinhardtii* como en las plantas existen pocos datos disponibles acerca de la regulación mitocondrial por luz. Las algas fotosintéticas tienen una característica adicional: son organismos unicelulares y están sometidos directamente a las condiciones ambientales. Probablemente, esto tiene efectos sobre la regulación celular, y también sobre la propia mitocondria.

Se ha propuesto que el alga incolora *Polytomella* sp. divergió del linaje de *C. reinhardtii* por la pérdida de un cloroplasto funcional (Round, 1980). Es así que supuestamente *Polytomella* sp. representa la adaptación de *C. reinhardtii* a condiciones no fotosintéticas. A diferencia del alga verde, *Polytomella* sp. puede crecer en un margen muy amplio de pH y puede utilizar diferentes fuentes de carbono (Wise, 1955). Sin embargo, ambas algas comparten algunas características en las mitocondrias, como el hecho de que la subunidad COXII del complejo IV está codificada en dos genes nucleares, cuyos productos proteicos se asocian para formar una subunidad madura. También se ha reportado que la subunidad β del complejo V en ambas algas es más grande que la α, debido a la presencia de una extensión en el extremo carboxilo-terminal (Franzén y Falk, 1992; Atteia y cols., 1997). Estas similitudes indican la cercanía evolutiva que tienen estas algas.

El estudio de la biogénesis, la composición mitocondrial, y la regulación en las dos algas debe contribuir al conocimiento de la ascendencia, evolución y función de las mitocondrias en relación con 1) la fotosíntesis, 2) la pérdida de la fotosíntesis, y 3) los organismos unicelulares.

OBJETIVOS

1. El primer objetivo de este trabajo consistió en comprender mejor la composición y la biogénesis de la mitocondrias del alga verde *C. reinhardtii* y del alga incolora *Polytomella* sp. Esto permite la comparación de las mitocondrias de las algas con las de plantas y animales, así como la comparación de las mitocondrias entre dos algas relacionadas evolutivamente. El cumplimiento de este objetivo es la base para estudios posteriores de regulación y evolución.

2. El segundo objetivo fue estudiar el papel que juegan las mitocondrias en las algas *C. reinhardtii* y *Polytomella* sp. durante su adaptación al medio ambiente. Quisimos estudiar la regulación mitocondrial por luz en *C. reinhardtii* y saber si las respuestas mitocondriales a la luz son comparables con las respuestas que presentan las plantas. En *Polytomella* sp., se puede estudiar la regulación mitocondrial en distintos medios de cultivo, variando el pH o la fuente de carbono.

RESULTADOS

TRABAJO PRINCIPAL : ARTÍCULO I

RESUMENES DE OTROS ARTÍCULOS PUBLICADOS O SOMETIDOS A PUBLICACIÓN

RESULTADOS NO PUBLICADOS

ARTÍCULO I

Identification of Novel Mitochondrial Protein Components of *Chlamydomonas reinhardtii*: A Proteomic Approach

van Lis, R., Atteia, A., Mendoza-Hernandez, G. and González-Halphen, D. (2003)

Plant Physiol 132, 318-330

RESUMEN

El proteoma mitocondrial del alga fotosintética *Chlamydomonas reinhardtii* fue analizado por electroforesis en geles azules nativos de poliacrilamida (BN-PAGE). Aplicando mitocondrias puras, se separaron los principales complejos de la fosforilación oxidativa (OXPHOS): la F_1F_o-ATP sintasa, la NADH-ubiquinona óxidorreductasa, la ubiquinol-citocromo *c* reductasa, y la citocromo *c* oxidasa. La F_1F_o-ATP sintasa siempre migró como un dímero, a diferencia de la CF_1CF_o-ATP sintasa del cloroplasto de *C. reinhardtii*. Se reportó la presencia de una nueva proteína que se asocia a la ATP sintasa y que podría estar involucrada en la dimerización de este complejo en *C. reinhardtii*. Los complejos de la OXPHOS separados por BN-PAGE fueron después separados en sus correspondientes subunidades por medio de geles desnaturalizantes de segunda dimensión (SDS-PAGE). Algunos polipéptidos fueron identificados por su secuencia amino terminal. Se analizaron las proteínas estructurales I y II del complejo III y se predijo sus actividades proteolíticas. Además, se demostró el carácter heterodimérico de la subunidad II de la citocromo *c* oxidasa. Se identificaron otras proteínas mitocondriales como la chaperonina HSP60, la oxidasa alterna, la aconitasa y el transportador de ADP/ATP.

Identification of Novel Mitochondrial Protein Components of *Chlamydomonas reinhardtii*. A Proteomic Approach[1]

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Pure mitochondria of the photosynthetic alga *Chlamydomonas reinhardtii* were analyzed using blue native-polyacrylamide gel electrophoresis (BN-PAGE). The major oxidative phosphorylation complexes were resolved: F_1F_0-ATP synthase, NADH-ubiquinone oxidoreductase, ubiquinol-cytochrome *c* reductase, and cytochrome *c* oxidase. The oligomeric states of these complexes were determined. The F_1F_0-ATP synthase runs exclusively as a dimer, in contrast to the *C. reinhardtii* chloroplast enzyme, which is present as a monomer and subcomplexes. The sequence of a 60-kD protein, associated with the mitochondrial ATP synthase and with no known counterpart in any other organism, is reported. This protein may be related to the strong dimeric character of the algal F_1F_0-ATP synthase. The oxidative phosphorylation complexes resolved by BN-PAGE were separated into their subunits by second dimension sodium dodecyl sulfate-PAGE. A number of polypeptides were identified mainly on the basis of their N-terminal sequence. Core I and II subunits of complex III were characterized, and their proteolytic activities were predicted. Also, the heterodimeric nature of COXIIA and COXIIB subunits in cytochrome *c* oxidase was demonstrated. Other mitochondrial proteins like the chaperone HSP60, the alternative oxidase, the aconitase, and the ADP/ATP carrier were identified. BN-PAGE was also used to approach the analysis of the major chloroplast protein complexes of *C. reinhardtii*.

The unicellular green alga *Chlamydomonas reinhardtii* is a model organism for the study of certain aspects of plant physiology, like chloroplast biogenesis (Harris, 2001). Nevertheless, *C. reinhardtii* mitochondria have not been well characterized because of difficulties in obtaining these organelles free of thylakoid contamination. The isolation of *C. reinhardtii* oxidative phosphorylation (OXPHOS) complexes, including the spectroscopical characterization of cytochrome bc_1 complex (complex III) and cytochrome *c* oxidase (complex IV), was described earlier (Atteia et al., 1992; Atteia, 1994). However, the subunit composition of the OXPHOS complexes in the alga has not been studied in detail.

The mitochondrial genome of *C. reinhardtii* encodes five subunits of complex I, cytochrome *b* of complex III, and subunit I of complex IV (Michaelis et al., 1990). Until now, none of these subunits have been located on SDS-PAGE. Among the mitochondrial proteins of nuclear origin, few have been identified and their genes sequenced: subunits alpha, beta, and ATP6 of complex V (F_1F_0-ATP synthase; Franzén and Falk, 1992; Nurani and Franzén, 1996, Funes et al., 2002), and two subunits of complex III, the Rieske-type iron-sulfur protein (Atteia and Franzén, 1996) and cytochrome c_1 (Atteia et al., 2002). The gene sequences of subunits COXIIA, COXIIB, and COXIII of the *C. reinhardtii* complex IV have been determined (Pérez-Martínez et al., 2000, 2001), but their protein products were not identified biochemically. Also, two genes encoding *C. reinhardtii* alternative oxidase (AOX), *Aox1* and *Aox2*, have been sequenced (Dinant et al., 2001). *Aox1*, the more expressed of the two genes, encodes a protein similar to plant AOXs, but lacks a conserved Cys residue at its N terminus. This Cys is thought to participate in the regulatory dimerization of the plant enzymes (Umbach and Siedow, 1993, 2000). The biochemical characterization of *C. reinhardtii* AOX remains to be addressed. Until now, validation of the information of the gene sequences by the analysis on the protein level has been largely missing for the mitochondrial proteins of this photosynthetic alga.

Blue native (BN)-PAGE is a powerful tool for proteomics. This technique uses the charge shift induced by the binding of Coomassie Blue to solubilized proteins to separate and visualize membrane complexes under native conditions (Schägger, 1995). BN-PAGE was developed to study protein complexes of bovine

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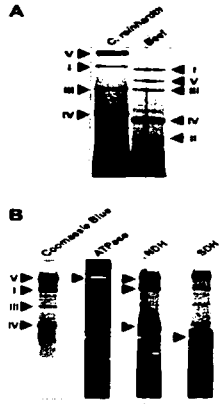

Figure 1. BN-PAGE of total mitochondrial proteins from *C. reinhardtii* and beef. A, Coomassie Blue-stained BN-PAGE gel lanes loaded with 800 (*C. reinhardtii* strain 84CW15) and 500 (beef) μg of total mitochondrial proteins. B, Gel lanes stained with Coomassie Blue and with specific activity stainings used for the detection of complexes V, I, and II (see "Materials and Methods"). Black arrows mark the major stained bands in each case. ATPase, ATPase activity; NDH, NADH dehydrogenase activity; SDH, succinate dehydrogenase activity.

mitochondria (Schägger and von Jagow, 1991) and later extended to study the mitochondrial complexes of yeast (*Saccharomyces cerevisiae*; Arnold et al., 1998), plants (Jänsch et al., 1996), and trypanosomatid kinetoplasts (Maslov et al., 1999). BN-PAGE has also been used to resolve chloroplast complexes of spinach (*Spinacia oleracea*; Kügler et al., 1997), mitochondrial complexes of Arabidopsis (Kruft et al., 2001), and simultaneously mitochondrial and chloroplast protein complexes of potato (*Solanum tuberosum*) leaves (Singh et al., 2000).

By applying pure mitochondria of *C. reinhardtii* (Eriksson et al., 1995) to BN-PAGE, we identified and characterized the OXPHOS complexes and their subunit composition. The oligomeric states of the complexes III to V and the AOX were analyzed. Finally, we used BN-PAGE to describe subcellular fractions containing both chloroplast and mitochondrial protein complexes from *C. reinhardtii* wild-type cells and from a photosynthetic mutant.

RESULTS

BN-PAGE of Mitochondrial Protein Complexes

To separate the major OXPHOS complexes, pure *C. reinhardtii* mitochondria (Eriksson et al., 1995) from the 84CW15 strain were solubilized and applied to BN-PAGE. The protein profile exhibited four major bands and several weaker bands (Fig. 1A) that differed from that of bovine heart mitochondria in the position, amount, and intensity of the bands. The apparent molecular masses of *C. reinhardtii* OXPHOS complexes were estimated from the known molecular masses of the bovine complexes and are summarized in Table I. The BN-PAGE profile of *C. reinhardtii* mitochondria exhibited two main characteristics: a band with considerably lower electrophoretic mobility than bovine complex I, and the absence of bands that correspond to the bovine complex V and complex II (Fig. 1A). To establish the identities of the *C. reinhardtii* major complexes, specific activity stainings were performed.

To localize the active *C. reinhardtii* complex V on BN-PAGE, a blue gel lane was incubated in the presence of ATP and $CaCl_2$. Figure 1B shows that the uppermost band of 1,600 kD was able to hydrolyze ATP, as indicated by the formation of a calcium phosphate precipitate. The high apparent molecular mass of complex V on BN-PAGE suggests that this protein complex runs as a dimer.

NADH dehydrogenase activity was detected after incubation of a blue gel lane in the presence of NADH and nitroblue tetrazolium (NBT), which forms a purple precipitate upon reduction. With *C. reinhardtii* mitochondria, three bands of approximately 1,500, 800, and 200 kD were detected (Fig. 1B). The thin band of 1,500 kD detected by the NADH/NBT staining was identified as a dimer of complex I. The 800-kD band, exhibiting an electrophoretic mobility similar to that of bovine complex I (Fig. 1A), was identified as a complex I monomer. Previously, complex I of *C. reinhardtii* was estimated to be 850 kD on BN-PAGE (Duby et al., 2001). The diffuse band of 200 kD (Fig. 1B) was also observed in the bovine protein pattern (not shown) and considered to be a complex I subcomplex.

Succinate dehydrogenase activity in the gel was visualized by the precipitation of reduced NBT in the presence of succinate and phenazine methosulphate.

Table I. *Estimated molecular masses of the respiratory complexes in C. reinhardtii and bovine mitochondria*

The molecular masses of the respiratory complexes of *C. reinhardtii* were estimated in comparison with the beef heart respiratory complexes reported earlier (Schägger and von Jagow, 1991).

Complex No.	Estimated Molecular Mass	
	C. reinhardtii	Beef
	kD	
V	1,600	600
I	800	750
III	500	500
IV	240	200
II	140*	130

* Based on complex II specific staining, shown in Fig. 1B.

Unlike bovine complex II, *C. reinhardtii* complex II did not appear as a defined band on the Coomassie Blue-stained gel, but as a diffuse band around 140 kD (Fig. 1B).

On the basis of their migrations and subunit composition (see below), which are comparable with the corresponding bovine complexes, the *C. reinhardtii* protein bands of 500 and 240 kD on BN-PAGE (Fig. 1A) were identified as complexes III and IV, respectively.

Resolution of *C. reinhardtii* OXPHOS Complexes into their Constitutive Subunits

C. reinhardtii mitochondrial complexes V, I, III, and IV, separated by BN-PAGE, were resolved into their individual constituents on second dimension (2D)-SDS-PAGE (Fig. 2). The estimated molecular masses of the subunits are given in Table II.

C. reinhardtii complex V was resolved into 13 polypeptides, three of which have been previously identified: the beta- (60 kD) and alpha- (52 kD) subunits of the F_1 sector (Atteia et al., 1992; Franzén and Falk, 1992; Nurani and Franzén, 1996) and the ATP6 subunit (21 kD) of the F_0 region (Funes et al., 2002). We determined the N-terminal sequence of the smallest polypeptide of 7 kD (Fig. 2; Table III, band 4). This N-terminal sequence was found to be encoded in the *C. reinhardtii* EST clone AW676361. The predicted protein corresponded to ATP9, a structural component of F_0-ATP synthase. Similarly, the N-terminal sequence of the 32-kD polypeptide (Table III, band 2) was found in the deduced amino acid sequence of EST clones BE337293 and AV390953 and allowed its identification as the gamma subunit (predicted molecular mass of 30.8 kD). Also, the N-terminal sequence of the 24-kD polypeptide of complex V (Table III, band 3) was found in the deduced protein sequence of EST clones AW661069 and BG848206, identified as the delta subunit (predicted molecular mass of 22.6 kD). Finally, the EST clones BI532011 and BG860760 were found to encode the previously determined N terminus of the 45-kD subunit of complex V (Funes et al., 2002), but the deduced partial amino acid sequence (165 amino acids) did not show similarity to any ATP synthase subunit.

When performing 2D-SDS-PAGE in the presence of 8 M urea, an additional 60-kD protein was resolved in the complex V polypeptide pattern. As shown in Figure 3, the 60-kD protein is not recognized by an anti-beta antibody. We determined the N-terminal sequence and an internal protein sequence of this polypeptide, here named MASAP (Table III, band 1). Subsequently, deoxyoligonucleotides were designed, a PCR product was obtained, and a corresponding cDNA was isolated from a λZAP cDNA library. From the deduced amino acid sequence, it was inferred that the MASAP is most likely soluble, exhibiting an apparent molecular mass of 60.5 kD and a pI of 5.66. No similarity to any mitochondrial protein in the databases was found. The protein presequence deduced from the cDNA was predicted to be mitochondrial using the TargetP V1.0 program (Emanuelsson et al., 2000). The function of this novel component remains to be established.

C. reinhardtii complex I (800-kD band on BN-PAGE) was resolved into at least 25 subunits on 2D-SDS-PAGE (Fig. 2). The N-terminal sequences of three of its constituents are reported in Table III (bands 5–7).

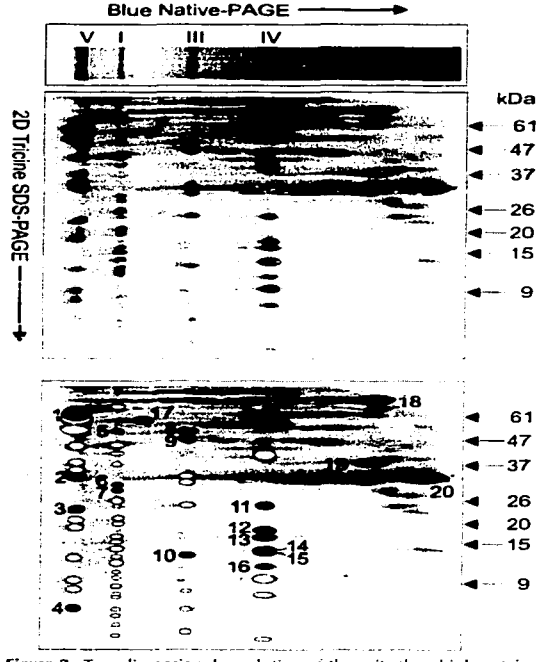

Figure 2. Two-dimensional resolution of the mitochondrial protein complexes from *C. reinhardtii*. The main OXPHOS complexes are indicated on the first dimension BN-PAGE. A BN gel lane was cut out and placed horizontally for subsequent resolution of the protein complexes into their respective components on 2D-Tricine-SDS-PAGE. In the schematic representation of the subunits (bottom), the numbered black spots depict those polypeptides that were subjected to Edman degradation. The corresponding sequences are shown in Table III. White spots represent the other putative subunits of each complex.

Table II. *Apparent number of subunits and the estimation of the molecular mass of the individual subunits of C. reinhardtii complexes V, I, III, and IV*

Complex No.	Apparent No. of Subunits[a]	Estimated Molecular Mass of Subunits
		kD
V	14	60,60,52,45,38,35,31.24,21,19,13,9,8,7
I	25	75,52,45,41,37,29,28.26,25,22,20,16,15,14,13,12,11,10,9,8,8,7,7,6,5
III	9	53,48,32,30,25,13,8,7,6
IV	10	40,25,18,16,14,14,12,10,8,5

[a] As detected by Coomassie Blue staining of Tricine SDS-polyacrylamide gel.

The 52-kD protein (band 5) of complex I exhibited an N-terminal sequence with an unusual high content of Pro. The EST clone AV386989 contained a sequence encoding the N terminus of this 52-kD protein, identified as a member of the 51-kD subunit family of complex I. Also, the EST clone BE212104 encoded the N-terminal sequence of the 28-kD subunit (band 7), a member of the 24-kD subunit family of complex I. Both the 51- and 24-kD subunit families are components of the flavoprotein fraction. The N-terminal sequence of the 29-kD subunit (band 6) was also found to be encoded in a clone of the ChlamyEST database (BM001979), but the deduced amino acid sequence did not allow its identification.

C. reinhardtii complex III was resolved on 2D-SDS-PAGE into nine subunits. The 53-kD subunit (Table III, band 8) was identified as the core I subunit by immunoblot analysis, using an antiserum against *Neurospora crassa* core I (see below). However, the N-terminal sequence of this band did not show any similarity with core I subunits from either plant or mammalian complex III. A clone from the EST database encoded the N-terminal sequence of this *C. reinhardtii* core I protein (BG846882). The whole sequence of core I was obtained from the overlapping EST clones BG846882, BI726156, AV633102, BG850841, and BG847806. The predicted mature core I protein (53.9 kD) contained 487 residues. The 48-kD protein of complex III (Fig. 2, band 9) is assumed to be the core II subunit, which probably comigrates with one or more proteins because a mixture of N-terminal sequences was obtained (not shown). In plants, cores I

Table III. *Partial description of the mitochondrial proteome of C. reinhardtii*

Amino acid sequences of the protein bands subjected to Edman degradation (see Fig. 2). GenBank accession nos. are provided. Alternatively, the accession nos. of the ChlamyEST database clones that were used to identify the proteins (expressed sequence [EST] in superscript) are given. NF, Not found in the ChlamyEST database.

Band No.	Amino Acid Sequence[a]	Protein Identity	Accession No.
Complex V			
1	YVTALKVEFS/ELAARSAEFRAEQEA (Int)	MASAP (60 kD)	AJ441255
2	ASNQAVKQRI/[Funes et al., 2002]	Gamma subunit (31 kD)	BE337293[EST]
3	AKTAPKAEM/[Funes et al., 2002]	Delta subunit (24 kD)	AW881069[EST]
4	SVLAASXMVGA	ATP9 subunit (7 kD)	AW678361[EST]
Complex I			
5	STAAPAAGAPPPPPPPAKT	51-kD subunit family (53 kD)	AV386989[EST]
6	VSSQFFDAPNGPSVKQVLIED	29-kD subunit (29 kD)	BM001979[EST]
7	ATNSTDIFNIHKDTPENNAA	24-kD subunit family (28 kD)	BE212104[EST]
Complex III			
8	QSAAKDVVATDANPFLRFSN	Core I (53 kD)	BG846882[EST]
9	More than one sequence	Probable core 2 + other protein(s) (48 kD)	
10	Blocked	Probable subunit IV (13 kD)	
Complex IV			
11	GSHAAGHQTAKEFYM	COXIII subunit (25 kD)	AAG17279
12	DAEVVEEEHAPPPPPPPKK	COXVIb subunit (18 kD)	BE122218[EST]
13	MDAVPX(G/R)LNQ	COXIIB subunit (16 kD)	AAK32114
14	GAPAEAKPSALSAEPGR	COXVb subunit (14 kD)	BG851120[EST]
15	DSPQPWQLLF	COXIIA subunit (14 kD)	AAK30367
16	ASTTAGETIDKY	COXVIa subunit (12 kD)	BG857268[EST]
Other proteins			
17	AAKDVRFGIEHRDLMLAGVNXLA	Mitochondrial HSP60 (60 kD)	NF
18	SXIAGAEKV(P/G)MSQFGP	Mitochondrial aconitate hydratase (90 kD)	AV397582[EST]
19	AAPSFGATRFXA	38-kD protein	NF
20	GIGECFVR (Int)	Mitochondrial ATP/ADP carrier (31 kD)	S30259

[a] Sequences are amino terminal unless mentioned otherwise (Int, internal).

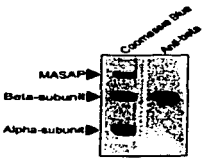

Figure 3. High-molecular mass subunits of *C. reinhardtii* complex V resolved on 2D-urea-SDS-PAGE. Complex V bands recovered from BN-PAGE were loaded onto a 2D-Tricine-SDS gel in the presence of 8 M urea. Only the largest subunits are shown. Left lane, Coomassie Blue staining; right lane, immunoblot analysis with an antibody against the beta-subunit. The immunoblot revealed that mitochondrial ATP synthase-associated protein (MASAP) is clearly distinct from the beta-subunit.

and II are known to represent the beta- and alpha-subunits of the mitochondrial processing peptidase (MPP), respectively. The alpha-MPP subunit does not possess MPP activity itself, but it is necessary for the beta-MPP activity. In most other organisms, the core proteins do not possess MPP activity, which is instead conferred by soluble, matrix-located alpha- and beta-MPP subunits (Braun and Schmitz, 1995a). The complete sequence of the core I of *C. reinhardtii* was analyzed for the presence of the consensus sequence for beta-MPP activity (Braun and Schmitz, 1995b). A multiple sequence alignment using core I and beta-MPP sequences (Fig. 4A) revealed that *C. reinhardtii* core I exhibits the consensus sequence, except for an Arg to Lys substitution at position 175. The Chlamy-EST database also allowed us to construct the sequence of *C. reinhardtii* core II, based on EST clones BM000676, AV631099, BI727574, and BM001151. This sequence exhibits similarity to core II and alpha-MPP subunits from other organisms, but lacks the consensus sequence for alpha-MPP activity (Fig. 4B).

In the 30-kD molecular mass range, *C. reinhardtii* complex III exhibits two subunits. Heme-specific 3,3′,5,5′-tetramethylbenzidine staining allowed the identification of the 30-kD protein as cytochrome c_1 (not shown). Thus, the 32-kD protein above the cytochrome c_1 is likely to be cytochrome *b*. The subunit of 25 kD was identified previously as the Rieske-type protein (Atteia and Franzén, 1996). The N terminus of the 13-kD subunit of complex III (Fig. 2, band 10) was not susceptible to Edman degradation.

Complex IV of the photosynthetic alga was resolved into 10 subunits. On the basis of its apparent molecular mass, the larger polypeptide (40 kD) was assigned as subunit I. The N-terminal sequences determined for the protein bands 11 (25 kD), 13 (16 kD), and 15 (14 kD) allowed their identification as subunits COXIII, COXIIB, and COXIIA, respectively (Pérez-Martínez et al., 2000, 2001). The N-terminal sequences of the proteins in bands 12 (18 kD), 14 (14 kD), and 16 (12 kD) were found to be encoded in the EST clones BE122218, BG851120, and BG857268, respectively. Homology searches led to the identification of band 12 as COXVIb (18 kD), band 13 as COXVb (14 kD), and band 14 as COXVIa (12 kD). In Figure 2, bands 14 and 15 were not resolved; however, the SDS-polyacrylamide gels used for N-terminal sequencing did allow the complete separation of these subunits.

Identification of Other Mitochondrial Proteins

The N-terminal sequences of other dominant proteins in *C. reinhardtii* mitochondria were also determined. The 38-kD protein (Fig. 2, band 19) could not be identified by its N-terminal sequence (Table III). The 31-kD protein (Fig. 2, band 20) was blocked at its N terminus. Nevertheless, the sequence of a tryptic fragment (Table III, band 20) matched a region from residues 54 to 61 of the *C. reinhardtii* ADP/ATP carrier (Sharpe and Day, 1993). The ADP/ATP translocator—as detected by Coomassie Blue staining—appeared to smear on BN-PAGE (Fig. 2).

The identity of the 60-kD protein (Table III, band 17) was established based on the similarity of its N-terminal sequence with that of mitochondrial chaperonin HSP60 (heat shock protein 60) of plants. On BN-PAGE, *C. reinhardtii* HSP60 was found to run as a faint band of approximately 650 kD (Fig. 2), indicating its multimeric nature. The HSP60 particle in the photosynthetic alga is probably a 14 mer, as in potato (Jänsch et al., 1996).

The N-terminal sequence of the 90-kD protein (Table III, band 18) was found to be encoded by an EST clone (AV397582) and corresponds to mitochondrial aconitate hydratase (aconitase). This soluble Krebs cycle enzyme that catalyzes the formation of isocitrate from citrate in the mitochondrial matrix appears to be a major constituent of the *C. reinhardtii* mitochondrial proteome. The entire amino acid sequence of the mature protein (776 residues, 83.2 kD) could be constructed on the basis of EST clones AV397582, AV631772, BI873612, BI873370, BF859712, and BF863471.

Oligomeric States of the OXPHOS Complexes

The oligomeric states of *C. reinhardtii* OXPHOS complexes were analyzed by immunoblot analysis of 2D-SDS-polyacrylamide gels subsequent to the application of pure mitochondria to BN-PAGE (Fig. 5). An antiserum against the beta-subunit of *Polytomella* sp. complex V recognized only the most upper band of BN-PAGE, previously identified as complex V (see above).

As revealed by immunoblot analysis with an antibody against *N. crassa* core I subunit, the major form of *C. reinhardtii* complex III was a dimer of 500 kD (Fig. 5). The antibody also recognized a minor form of 1,000 kD.

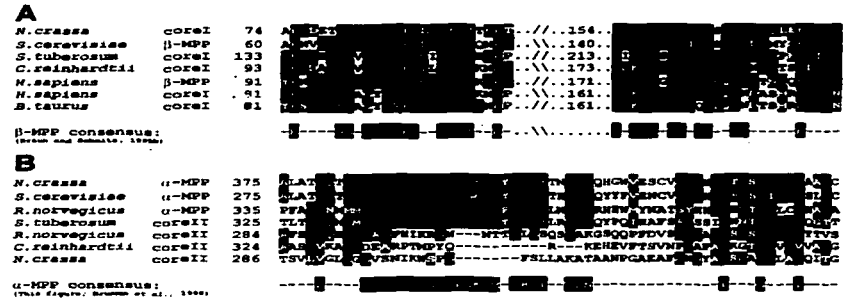

Figure 4. Multiple sequence alignments of the core I and II proteins and the MPP subunits from various sources. The accession number for each sequence is shown on the right-hand side. The *C. reinhardtii* sequences were derived from the EST clones indicated in the text. A, Comparison of *C. reinhardtii* core I with other core I and beta-MPP amino acid sequences. *C. reinhardtii* core I exhibits the consensus sequence usually found for beta-MPP protease activity, including the zinc-binding motif (H-X-X-E-H) that is absent in the mammalian core I sequences. B, Alignment of core II and alpha-MPP amino acid sequences. The core II sequence of *C. reinhardtii* lacks the consensus sequence (G-G-G-G-S-F-S-A-G-G-P-G-K-G-M-S-R-L-Y) believed to be required for alpha-MPP activity.

C. reinhardtii complex IV was detected immunochemically with an antibody against the COXIIB subunit of *Polytomella* sp. and appeared to be present in BN-PAGE in several oligomeric states, with apparent molecular masses of 530, 240, and 160 kD (Fig. 5). The 240-kD form was the most abundant.

In the absence of a specific antibody for complex I, it could nonetheless be inferred from Figure 1B that a minor fraction of complex I runs as a dimer. A BN-PAGE band of 1,500 kD was reproducibly detected by the specific staining for NADH dehydrogenase activity. Furthermore, on 2D SDS-PAGE, the polypeptide pattern of this high-molecular mass complex seemed to be identical to that of complex I, although this cannot be clearly discerned in Figure 2 due to its low abundance and proximity to complex V.

C. reinhardtii AOX

AOX is a mitochondrial key enzyme in photosynthetic organisms (Vanlerberghe and McIntosh, 1997). In the BN-PAGE analyses of plant mitochondria reported so far, no mention of the AOX has been made. In this study, antibodies were raised against the overexpressed C terminus of *C. reinhardtii* AOX1 and used to localize the corresponding protein. Immunoblots of 2D-SDS-polyacrylamide gels revealed the presence of the 36-kD AOX all over the width of the gel (Fig. 5). In contrast to the other respiratory complexes, *C reinhardtii* AOX was not resolved as a discrete band under the conditions used (2 mg *n*-dodecyl maltoside mg^{-1} mitochondrial protein). The behavior of the AOX on BN-PAGE is likely due to its propensity to form aggregates (Berthold and Siedow, 1993). At this stage, it is not known whether BN-PAGE is suitable to obtain a good resolution of the AOX protein or of other membrane-bound proteins such as the ADP/ATP carrier.

A Proteomic Approach to the Analysis of Subcellular Fractions, Different Growth Conditions, and Mutants

We have explored different uses of BN-PAGE for the comprehensive characterization of *C. reinhardtii* mitochondrial protein components. The purification procedure of *C. reinhardtii* mitochondria consists of cell rupture, two differential centrifugations, and a Percoll gradient centrifugation step that removes remnant chloroplast proteins (Eriksson et al., 1995). To follow the enrichment of mitochondria during this procedure, the pellets of the two differential centrifugations (P1 and P2) were analyzed on BN-PAGE (Fig. 6). In pellet P1, resulting from the centrifugation of the cell homogenate at 2,000*g*, the photosynthetic complexes were dominantly present (Fig. 6A). The distribution of these complexes on BN-PAGE is roughly comparable with that of spinach (Kügler et al., 1997) and potato chloroplast complexes (Singh et al., 2000). PSII (300 kD) was identified by immunoblotting with an antibody against the D1 protein (not shown). The chloroplast ATP synthase (CF_0CF_1-ATP synthase) was identified by its typical subunit composition. Apart from the monomer of 500 kD (Fiedler et al., 1995), at least three subcomplexes of CF_0CF_1-ATP synthase could be separated on BN-PAGE (Fig. 6), including the CF_1 entity of approximately 350 kD. In pellet P1, mitochondrial complexes V, I, and IV could be detected by Coomassie Blue staining. Figure

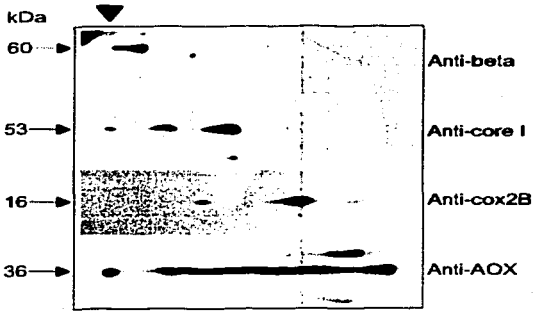

Figure 5. Oligomeric states of mitochondrial protein complexes as detected by immunoblotting. Proteins resolved by 2D-Tricine-SDS-PAGE gels were transferred onto nitrocellulose membranes and immunoblotted with the indicated antibodies (from top to bottom, anti-beta subunit of *Polytomella* sp. ATP synthase, anticore I of *N. crassa*, anti-COXIIB of *Polytomella* sp., and anti-AOX of *C. reinhardtii*). The arrow indicates the position of the well on the first dimension BN-PAGE, where a small portion of total proteins precipitates before entering the stacking gel.

6A reveals the great contrast in the electrophoretic behavior between chloroplast and mitochondrial ATP synthases in the green alga. Although several chloroplast ATP synthase oligomeric forms and subcomplexes were visible, only a single, high molecular form of the mitochondrial enzyme was observed. Pellet P2 represents the crude mitochondrial fraction that results from the second centrifugation step at 3,000*g* and shows a pronounced enrichment in mitochondrial protein complexes. Complexes V, I, and IV were clearly visible, whereas complex III was obscured by the chloroplast ATP synthase and PSI (Fig. 6B). Pure mitochondria were obtained after Percoll density gradient centrifugation and are typified by the virtual absence of chloroplast protein complexes (Fig. 6C).

We also analyzed the crude mitochondria of the photosynthetic mutant strain BF4.F54.F14 (Fig. 6D, comparable with fraction P2 in Fig. 6B). This mutant is devoid of PSI, CF_0CF_1-ATP synthase (Chua et al., 1975; Piccioni et al., 1981), and most of the light-harvesting complexes (Olive et al., 1981). To obtain mitochondria from this cell wall-containing strain, the cells were pretreated with CTAB. As expected, the only photosynthetic complexes found in the crude mitochondria were the b_6f complex and PS II. No differences in the mitochondrial protein patterns were observed between the mutant strain and the wild-type strain. Nevertheless, in the mutant, the mitochondrial complex III subunits appeared clearly on the 2D gels (Fig. 6C, arrow).

DISCUSSION

The Electron Transfer Complexes and Their Oligomeric States

Previous works have analyzed the mitochondrial proteome of the model plant Arabidopsis (Kruft et al., 2001; Millar et al., 2001). Besides land plants (*Streptophyta*), green algae (*Chlorophyta*) are the other main constituent of *Chlorobionta*. In this work, we addressed the study of mitochondria from *C. reinhardtii*, a unicellular model system for photosynthetic cells. To characterize the mitochondria of *C. reinhardtii*, we used BN-PAGE, a powerful analytical technique for both membrane and soluble proteins. A critical parameter to study the mitochondrial proteome is the purity of the sample to be analyzed. *C. reinhardtii* intact mitochondria were prepared according to Eriksson et al. (1995). These mitochondria were assessed to be basically free of chloroplast contamination by comparing the 2D-SDS-PAGE polypeptide pattern of the different fractions obtained during the purification procedure (Fig. 6).

The estimation of the molecular mass of proteins from their migration on BN-PAGE is approximate because this technique separates according to size but also according to charge (Schägger and von Jagow, 1991). It was inferred that the behavior of OXPHOS complexes on BN-PAGE resembles their physiological state in the mitochondrial inner membrane at the time of solubilization. For yeast and mammalian mitochondria, when low detergent to protein ratios were used for solubilization, the association of different protein complexes in supercomplexes was revealed (Schägger and Pfeiffer, 2000). These complex-complex interactions seem to reflect functional associations that exist in vivo, the so-called respirosome.

The resolution of the mitochondrial protein complexes of *C. reinhardtii* in BN-PAGE was clearly distinct from the pattern obtained with Arabidopsis mitochondria (Kruft et al., 2001). In all BN-PAGE experiments, *C. reinhardtii* complex I was found to run mainly as a monomer. Two other forms could be detected by activity staining: a minor form of high molecular mass (1,500 kD) that probably corresponds to a dimer and a subcomplex of 200 kD. In agreement with the results of Cardol et al. (2002), the 200-kD band represents a soluble fraction that contains the hydrophilic 49- and 76-kD subunits of the complex I peripheral arm. It is likely that the complex I monomer represents the physiological state of this protein in mitochondria because even in the most mild solubilization conditions, it was always found as a monomer (Schägger and Pfeiffer, 2000). In addition, complex I has been shown to associate with complexes III and IV (Schägger and Pfeiffer, 2000). Nevertheless, the 1,500-kD band in *C. reinhardtii* is thought to represent only dimeric complex I because immunoblot analysis of 2D-SDS-PAGE with antibod-

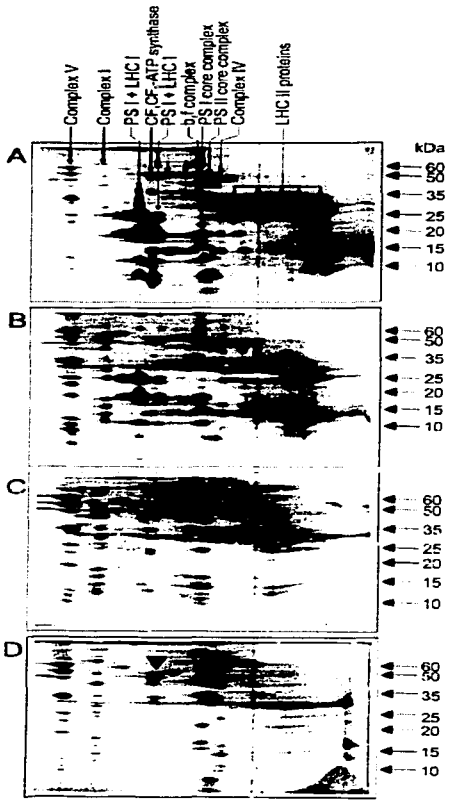

Figure 6. 2D-Gly SDS-polyacrylamide gels comparing different fractions of the isolation procedure for mitochondria from the *C. reinhardtii* 84CW15 strain and the photosynthetic mutant BF4.F54.F14. The indicated sample (350 μg total protein) was subjected to BN-PAGE and to subsequent denaturing 2D. The main mitochondrial and photosynthetic complexes are indicated by arrows. LHC I and II, Light-harvesting complex I and II; CF_1CF_0 ATP synthase, chloroplast ATP synthase. The first three panels correspond to fractions of the *C. reinhardtii* 84CW15 strain. A, P1, the first pellet after cell disruption and centrifugation at 2,000*g*. B, P2, Pellet obtained after centrifugation at 5,000*g* of the supernatant resulting from the first centrifugation that constitutes the crude mitochondrial fraction. C, Mitochondria, purified by Percoll density gradient centrifugation. D, Pellet P2 from the triple photosynthetic mutant BF4.F54.F14. This mutant was treated with *N*-cetyltrimethylammonium bromide (CTAB) to enable cell rupture by glass beads, as indicated in "Materials and Methods." The black bold arrow indicates the position of complex III.

ies against subunits of complexes III and IV (Fig. 5) never indicated the presence of supercomplexes. The possible physiological role of dimeric complex I remains to be established.

Immunoblot analysis allowed the identification of oligomeric forms of the respiratory complexes III and IV (Fig. 5). The major form of complex III is a dimer of 500 kD coexisting with a minor form of 1,000 kD. In other organisms, complex III is mainly present as a dimer as well. It was found that the beef complex III dimer is more active than the monomer (Nalecz and Azzi, 1985). In addition, cytochrome *c* binds to only one recognition site of the dimeric yeast bc_1 complex (Lange and Hunte, 2002), and the dimeric yeast bc_1 complex oxidizes ubiquinol by an alternating, half-of-the-sites mechanism (Gutiérrez-Cirlos and Trumpower, 2002).

Antibodies against the COXIIB subunit of the colorless *C. reinhardtii* relative *Polytomella* sp. showed that *C. reinhardtii* complex IV is present mainly in a 240-kD form. In potato, the 160-kD monomeric form was predominant, but a portion of 230 kD was also present (Jänsch et al., 1996). The 240-kD form in *C. reinhardtii* is smaller than the theoretical dimer (300 kD) and may represent a dimeric cytochrome *c* oxidase exhibiting anomalous migration in BN-PAGE. The crystal structure of beef complex IV is clearly dimeric (Tsukihara et al., 1996), although solubilized dimers are difficult to maintain and easily dissociate into monomers (Musatov et al., 2000). Also, monomers have been reported to be more active than dimers (Nalecz et al., 1983).

Although BN-PAGE allowed the high resolution of several *C. reinhardtii* OXPHOS complexes, other proteins, such as complex II, the AOX, and the ADP/ATP carrier, ran as diffuse bands or smeared along the gel. The pattern on 2D-SDS-PAGE for the ADP/ATP carrier suggested that it was present on the first dimension either in multiple oligomeric forms, as partial aggregates, or both (Fig. 2). The AOX, a membrane-bound protein, also aggregates under the electrophoretic conditions applied. Surprisingly, the same is true for the aconitase, which is clearly a soluble protein. The high resolution of some complexes, along with the aggregation of some other proteins under the same conditions, might be an inherent property of BN-PAGE. With this technique, it was claimed that several mitochondrial dehydrogenases in yeast form supramolecular complexes (Grandier-Vazeille et al., 2001). However, care must be taken to distinguish supercomplexes from contamination that originates from smeared proteins. The comigration of proteins in discrete regions of BN-PAGE may reflect a contribution of aggregates

and not necessarily indicate in vivo associations. The associations should exhibit a certain stoichiometry, and the conclusions should be corroborated using an independent method, i.e. cross-linking, gradient centrifugation, or gel filtration experiments.

C. reinhardtii Mitochondrial Complex V Is Atypical

C. reinhardtii complex V is resolved on 2D-SDS-PAGE into at least 13 distinct subunits (Funes et al., 2002; this work), comparable with 13 subunits in beef (Schägger and von Jagow, 1991), in potato (Jänsch et al., 1996), in Arabidopsis (Kruft et al., 2001), and in *Polytomella* sp. (Atteia et al., 1997; A. Atteia and R. van Lis, unpublished data). This work allowed the identification of subunits gamma (31 kD), delta (24 kD), and ATP9 (7 kD). These subunits do not exhibit amino acid extensions as do the alpha- and beta-subunits (Atteia et al., 1992; Franzén and Falk, 1992; Nurani and Franzén, 1996). In contrast to mitochondrial ATP synthases from plant or mammalian sources, the gamma-subunit in *C. reinhardtii* (31 kD) is not the third largest protein of the complex because three unidentified proteins of 45, 38, and 35 kD were present in the polypeptide pattern of complex V.

When using 2D-SDS-PAGE supplemented with 8 M urea, an additional 60-kD polypeptide was resolved from *C. reinhardtii* complex V separated on BN-PAGE. This polypeptide, named MASAP, was previously found to be associated with *C. reinhardtii* complex V isolated by Suc density gradients (Atteia, 1994). Because solubilization was performed with 5% (w/v) Triton X-100 and the gradients contained 0.5 M potassium phosphate and 0.2% (w/v) Triton X-100, it can be concluded the MASAP tightly interacts with complex V. The previously reported N-terminal amino acid sequence of MASAP (Atteia, 1994) was confirmed in this work, and the complete sequence of the corresponding cDNA was obtained. The deduced amino acid sequence did not show similarity to other mitochondrial proteins in the databases. Yet, its presequence has all the characteristics of a mitochondrial targeting sequence. A 66-kD protein, identified as the HSP66 chaperonin, has been found associated to yeast ATP synthase (Gray et al., 1990). However, MASAP does not show any similarity to heat shock proteins, making it unlikely to be a chaperonin.

Assuming that the 14 proteins in *C. reinhardtii* are genuine constituents of complex V, the expected monomer of this complex would be 740 kD. Nevertheless, this complex exhibited the lowest electrophoretic mobility on BN-PAGE with an estimated molecular mass of 1,600 kD. In contrast, monomeric complex V from yeast, plants, and mammals has a molecular mass of 550 to 580 kD on BN-PAGE (Schägger, 1995; Jänsch et al., 1996; Arnold et al., 1998; Kruft et al., 2001). Also, the *C. reinhardtii* chloroplast ATP synthase exhibited a molecular mass of 500 kD (Fig. 6). On the same gels, the mitochondrial and chloroplast ATP synthases of the green alga clearly behaved differently. In addition, both specific staining and immunolabeling could not reveal the presence of a mitochondrial F_1-ATP synthase moiety. This also contrasts with BN-PAGE analysis of plant, trypanosomatid, and mammalian mitochondria, which invariably revealed the presence of dissociated F_1-ATP synthase particles (Schägger and von Jagow, 1991; Jänsch et al., 1996; Kügler et al., 1997; Maslov et al., 1999; Singh et al., 2000; Kruft et al., 2001). Clearly, the behavior of *C. reinhardtii* complex V on BN-PAGE differs from the ones observed in other organisms.

Complex V dimers have been observed on BN-PAGE with mammalian and yeast mitochondria but only as a small fraction of the total amount. In the case of yeast complex V, dimeric forms were observed when mitochondrial membranes were solubilized with low detergent to protein ratios. Three additional small subunits—g, h, and Tim 11—are believed to be involved in the dimerization of the yeast complex (Arnold et al., 1998). Altogether, our data strongly suggest an unprecedented strong dimerization of *C. reinhardtii* mitochondrial complex V and an uncommon resistance to dissociation of the F_1 sector. We hypothesize that MASAP, by itself or in conjunction with the three unidentified proteins of 45, 38, and 35 kD, participate in the formation of highly stable complex V dimers in *C. reinhardtii*. Also, the unique amino acid extensions identified in the alpha- and beta-subunits (Franzén and Falk, 1992; Nurani and Franzén, 1996) could play a role in the dimerization of complex V.

The Core Proteins in *C. reinhardtii* Complex III

In eukaryotes, complex III has core I and core II subunits, two mitochondrial matrix-exposed proteins not involved in electron transfer. In plants, these proteins function as a MPP, and may have originated from a protease that was integrated into the bc_1 complex during early stages of the endosymbiotic event that gave rise to mitochondria (Braun and Schmitz, 1995b). In contrast to plants, the MPP activity in the photosynthetic alga *C. reinhardtii* was shown to be soluble (Nurani et al., 1997). Also, complex III of *Polytomella* sp., a non-photosynthetic relative of *C. reinhardtii*, is proteolytically inactive (Brumme et al., 1998). In this work, we identified *C. reinhardtii* core I subunit and determined its complete sequence using the ChlamyEST database. The deduced protein exhibits similarity to beta-MPP and core I subunits from different organisms. Core I exhibits the complete inverse zinc-binding motif (HXXEH), which was shown to be essential for the proteolytic activity of MPP in rat mitochondria (Kitada et al., 1995). The core I of *C. reinhardtii* has the beta-MPP consensus sequence (Braun and Schmitz, 1995b), except for a single Arg to Lys substitution at position 175. However, this substitution is unlikely to be responsible of

the lack a beta-MPP activity. In addition, the proposed core II sequence derived from the ChlamyEST database did not exhibit the consensus sequences for alpha-MPP. This raises the possibility that the MPP activity in *C. reinhardtii* could be organized as in *N. crassa* (Hawlitschek et al., 1988), with the core I protein exhibiting beta-MPP activity and the alpha-MPP being a soluble protein in the mitochondrial matrix. In the study of Nurani et al. (1997), the soluble fraction of *C. reinhardtii* was shown to exhibit proteolytic activity. It is likely that the preparation of this soluble fraction by sonication might have caused a certain level of dissociation of the core I subunit from complex III, giving rise to the observed soluble MPP activity.

C. reinhardtii Complex IV

This work provides new insights into the subunit composition of complex IV of the photosynthetic alga. The identification of COXIIA and COXIIB as distinct subunits of 14 and 16 kD indicates that the *C. reinhardtii* subunit COXII is a heterodimer, as previously shown for *Polytomella* sp. (Pérez-Martínez et al., 2001). In contrast to *Polytomella* sp., *C. reinhardtii* COXIIA and COXIIB subunits are well separated on 15% (w/v) Tricine-SDS polyacrylamide gels. The N-terminal sequence of *C. reinhardtii* COXIII and COXIIA determined in this study confirmed the prediction of the cleavage site in the preproteins. However, the sequence determined for COXIIB does not coincide with the N terminus predicted from the gene (Pérez-Martínez et al., 2001). This sequence was found to correspond to an internal sequence starting at residue 96 of the deduced mature protein. The same internal sequence was determined for COXIIB from *Polytomella* sp. (Pérez-Martínez et al., 2001). It was suggested that the COXIIB N terminus is blocked and that the observed sequence represents a region of the protein that is cleaved during Edman degradation. Three additional subunits of *C. reinhardtii* complex IV (COXVIb, COXVIa, and COXVb) were also identified. COXVb sequence is atypical because its first 40 residues and the last 40 residues show very poor similarity with is mammalian counterparts. Also, the first 60 residues of *C. reinhardtii* mature COXVIb did not show any similarity to other COXVIb subunits; this extension accounts for the fact that the green algal COXVIb has a molecular mass at least twice that of typical COXVIb subunits. The N-terminal sequence of COXVIb is characterized by a high content of Pro and charged residues, with a highly acidic theoretical pI of 4.39. The atypical sequences of some constituents of *C. reinhardtii* complex IV raise questions on the assembly and interactions of the complex IV subunits in the inner mitochondrial membrane.

Toward Functional Proteomics

The application of different subcellular fractions to BN-PAGE, either membranous, soluble, or whole organelles, enables a comprehensive study of the effect of growth conditions, mutations, and other factors that can influence biogenesis and metabolism. This is exemplified by the resolution of the chloroplast complexes together with their mitochondrial counterparts and by the analysis of the BF4.F54.F14 mutant strain. Among the many mutant strains available in *C. reinhardtii*, only few have been characterized at the biochemical level (de Vitry and Vallon, 1999; Duby et al., 2001). The impact of mutations in nuclear and organellar genes is likely to be better understood using a proteomic approach. The method developed in this work to isolate intact mitochondria from strains that have cell walls using CTAB makes BN-PAGE studies amenable for any *C. reinhardtii* mutant or wild-type strain.

We presented a partial catalog of the *C. reinhardtii* mitochondrial proteome based on BN-PAGE. With this approach, the behavior and composition of protein complexes was revealed, novel proteins were described (MASAP), some unusual structural features of proteins encoded by previously characterized genes were demonstrated (COXIIA and COXIIB), and novel predictions were made based on newly obtained sequences (cores I and II). With the genome project of *C. reinhardtii* approaching finalization, a more complete picture of the mitochondrial proteome may be obtained.

MATERIALS AND METHODS

Cell Growth and Isolation of Mitochondria

All *Chlamydomonas reinhardtii* strains were grown at 25°C to 26°C in Tris-acetate phosphate medium (Harris, 1989) in continuous light and agitation. For the cell wall-less strain 84CW15, the medium was supplemented with 1% (w/v) sorbitol. Mitochondria from 84CW15 cells were isolated in their late exponential growth phase as described by Eriksson et al. (1995). To isolate mitochondria from strains containing cell walls, the cells were resuspended in washing buffer (20 mM HEPES [pH 7.2]) to a concentration of 50 mg wet weight mL^{-1}. Subsequently, 50 μM CTAB was added from a 10 mM stock solution, and the cells were incubated at room temperature with agitation for 5 min. Before cell disruption with glass beads, the cells were diluted 5-fold and washed twice in washing buffer. The major portion of the orange precipitate that formed on top of the pellet of the second centrifugation of the mitochondrial purification procedure (Eriksson et al., 1995) was removed by pipetting and discarded; this enabled the application of the sample to BN-PAGE.

BN-PAGE

Sample preparation and BN-PAGE were carried out as described by Schägger and von Jagow (1991) with the following modifications: Isolated mitochondria or other cell fractions were first washed with 0.25 M sorbitol and 15 mM Bis-Tris (pH 7.0) and then resuspended in sample buffer (50 mM Bis-Tris and 0.75 M amino caproic acid [pH 7.0]). Pure mitochondria (final protein concentration of 5 mg mL^{-1}) was solubilized in the presence of 1% (w/v) *n*-dodecyl maltoside. Other fractions were solubilized in the presence of 2% (w/v) *n*-dodecyl maltoside at the same protein concentration. From mitochondrial fractions of cell wall-containing strains that were pretreated with CTAB, any residual orange precipitates were removed during the

washing steps and also after solubilization. The solubilization was carried out with samples prepared the same day. Once solubilized, the proteins could be stored on ice at 4°C up to a week. Linear polyacrylamide gradients varied from 5% to 10% to 5% to 15% (w/v). To minimize protein aggregation in the sample wells or in the gel, the stacking gel was poured immediately onto the resolving gel before it polymerized. For electrophoresis, either the Vertical Gel Electrophoresis System VI6 (4–5-h run at 20–25 mA; Gibco-BRL, Cleveland) or the Bio-Rad Mini Protean II system (1 h run at 15 mA; Bio-Rad Laboratories, Hercules, CA) was used. No prerun was performed.

Specific Staining of the OXPHOS Complexes

Covalently linked hemes were detected on SDS-polyacrylamide gels by their peroxidase activity in the presence of 3,3',5,5'-tetramethylbenzidine (Thomas et al., 1976). Other specific stainings were carried out directly on the blue gel lanes. NADH dehydrogenase activity was detected in 100 mM Tris-HCl (pH 7.4) containing 1 mg mL^{-1} NBT and 100 mM NADH (Kuonen et al., 1986). Succinate dehydrogenase activity was assayed in a buffer containing 50 mM phosphate buffer (pH 7.4), 100 mM sodium succinate, 200 μM phenazine methosulphate, and 2 mg mL^{-1} NBT (Jung et al., 2000). ATPase activity was located in situ by the method of Horak and Hill (1972), incubating the lane of BN-PAGE overnight in 10 mM ATP and 30 mM $CaCl_2$ in 50 mM HEPES (pH 8.0).

2D-Tricine-SDS-PAGE

Entire lanes from BN-PAGE were used to resolve the subunits in the 2D-Tricine-SDS-PAGE (15% [w/v] acrylamide) as described by Schägger and von Jagow (1991). Alternatively, Gly-SDS-PAGE (15% [w/v] acrylamide) was used (Laemmli, 1970). Where indicated, 2D-Tricine-SDS-PAGE was run in the presence of 8 M urea. Apparent molecular masses were estimated using BenchMark protein standards (Invitrogen, Carlsbad, CA).

Protein Analysis

Protein concentrations were determined as described by Markwell et al. (1978). Samples containing chlorophyll were precipitated using methanol and chloroform (Wessel and Flugge, 1984) before protein determination. After electrophoresis, proteins were electrotransferred onto nitrocellulose (Bio-Rad) or ProBlot membranes (Amersham-Pharmacia Biotech, Uppsala) using 50 mM H_3BO_3 and 50 mM Tris (no pH adjustment) as transfer buffer (tank transfer system). Immunodetection was carried out using the ECL kit (Amersham-Pharmacia Biotech) or the Pico kit (Pierce Chemical, Rockford, IL). The antisera used were raised against *C. reinhardtii* AOX (see below), *Neurospora crassa* core I subunit, and the COXIIB and beta-ATP synthase subunits of *Polytomella* sp. For N-terminal sequencing, the bands of the protein complexes resolved by BN-PAGE were excised from preparative gels. The slices were incubated in cathode buffer containing 1% (v/v) β-mercaptoethanol for 20 min, rinsed with cathode buffer, and loaded as a stack on top of a Tricine-SDS-PAGE. N-terminal analysis of electroblotted proteins onto polyvinylidene difluoride membranes was performed by automated Edman degradation at the Faculty of Medicine, Universidad Nacional Autónoma de México (LF 3000 Beckman sequencer, Beckman Instruments, Fullerton, CA) or at the Institut Pasteur, Paris (Procise 494 or 473A sequencers, PE-Applied Biosystems, Foster City, CA), all equipped with on-line HPLC apparatus. Internal sequencing after trypsinolysis was carried out as previously described (Atteia et al., 1997).

Cloning of the cDNA Encoding the MASAP

Using the degenerate oligodeoxynucleotides 5'-TAC GT(G/C) AC(G/C) GC(G/C) CT(G/C) AAC G-3' and 5'-CTC CTG CTC (G/C) GC (G/C) C GCA AC-3', designed on the N-terminal and internal amino acid sequences of MASAP, a PCR product of 1,173 bp was obtained using as template a mass excision plasmid preparation from a λZAP II cDNA library of *C. reinhardtii*. Samples were denatured for 5 min at 95°C and subjected to three cycles of 30-s denaturation at 95°C, 40 s of annealing at 62°C, and 2-min extension at 72°C, followed by 32 cycles of 30-s denaturation at 95°C, 40 s of annealing at 64°C, 2-min extension at 72°C, and a last 10-min extension at 72°C. The fragment was cloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced using the T7 and SP6 primers. The amplified DNA fragment was used to screen the cDNA library of *C. reinhardtii*. A cDNA of 2.4 kb was obtained and sequenced. The complete sequence is available at the GenBank/EBI Data Bank (accession no. AJ441255).

AOX Carboxy Terminus Overexpression and Antibody Production

Primers were designed based on the sequence of the *C. reinhardtii Aox1* gene (accession no. AF352435): 5'-GAC GAG CTC CTG CTG TCG CCG CGC AC-3' and 5'-CTG AAG CTT GGG CAG CTG GCT GGC GC-3'. Underlined are the added *Sac*I and *Hind*III restriction sites. PCR amplification with *Taq* polymerase was done using as a template a plasmid preparation obtained by mass excision from a λ ZAPII *C. reinhardtii* cDNA library. Samples were denatured for 5 min at 95°C and subjected to three cycles of 30-s denaturation at 95°C, 40 s of annealing at 62°C, and 1-min extension at 72°C, followed by 32 cycles of 30-s denaturation at 95°C, 40 s of annealing at 64°C, and 1-min extension at 72°C and a last 10-min extension at 72°C. The 360-bp product was cloned into the restriction sites *Sac*I and *Hind*III of the pQE30 vector (Qiagen USA, Valencia, CA), and the C-terminal region of the AOX protein of 11 kD containing a six-residue His tag was overexpressed and purified using a nickel-nitrilotriacetic acid agarose resin according to the manufacturer's instructions. The purified overexpressed C-terminal AOX fragment was used to raise antibodies in a rabbit.

Sequence Analysis in Silico

Protein sequences were obtained from ENTREZ at the NCBI server (www.ncbi.nlm.nih.gov), and alignments were made with the FASTA program (vega.igh.cnrs.fr/bin/fasta-guess.cgi). EST clones of *C. reinhardtii* were obtained from the ChlamyEST database (http:/www.biology.duke.edu/chalmy) using the WU-TBLASTN program. Multiple alignments were done with ClustalW (Thompson et al., 1994; searchlauncher.bcm.tmc.edu). Molecular masses and pI calculations were done with the compute pI/molecular mass tool (Bjellqvist et al., 1993), and the prediction of intracellular sorting was done with the TargetP V1.0 program (Emanuelsson et al., 2000), both from the ExPASy Molecular Biology Server (www.expasy.ch).

Note added in proofs

Recent data on the bovine heart complex I indicate a molecular mass of over 900 kDa instead of 750 kDa, as mentioned in this work (Carroll et al., 2002). This would modify the estimated molecular masses of the bands on BN-PAGE, corresponding to the *C. reinhardtii* complexes I and V, to around 1000 kDa and 2000 kDa, respectively.

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ARTÍCULO II

***Polytomella* sp. growth on ethanol.**
Extracellular pH affects the accumulation of mitochondrial cytochrome c_{550}.

Atteia, A., van Lis, R., Ramírez, J. and González-Halphen, D. (2000)

Eur J Biochem 267, 2850-2858

RESUMEN

El alga incolora *Polytomella* sp. crece en etanol como única fuente de carbono a pH ácido; obteniéndose mayores densidades celulares a un pH cercano a 3.7. El análisis espectrofotométrico de las células crecidas en etanol indicó que la intensidad del pico de absorbencia a 552 nm (citocromos de tipo *c*) es mayor en las células crecidas a pH 3.7 que en las crecidas a pH 6.0. En contraste, no se observaron cambios en la intensidad de las bandas α de los citocromos tipo *b* (567nm) y *a* (606nm). En geles de poliacrilamida-SDS teñidos con TMBZ, se observó una mayor acumulación de un citocromo *c* de bajo peso molecular (12kDa) en las células crecidas a pH 3.7. Tomando en cuenta que : 1) este citocromo presenta una reacción cruzada con un anticuerpo dirigido contra el citocromo *c* soluble de *C. reinhardtii*; 2) la secuencia de un fragmento interno del citocromo de *Polytomella* sp. se encuentra en los citocromos *c* mitocondriales; 3) los espectros absolutos (oxidado, reducido con ascorbato) de este citocromo purificado son típicos de los citocromos *c* solubles, se concluyó que el pH extracelular afecta el nivel de acumulación del citocromo *c* mitocondrial en el alga incolora *Polytomella* sp. Un experimento de hibridización tipo Northern utilizando como sonda el DNA que codifica al citocromo *c* mitocondrial de *C. reinhardtii (cyc)*, reveló una mayor acumulación del mRNA-*cyc* en las células crecidas a pH 3.7 que en las células crecidas a pH 6.0. Se aislaron mitocondrias de las células de cada cultivo y se hicieron estudios del consumo de oxígeno en presencia o en ausencia de inhibidores de transportadores de electrones de la cadena respiratoria clásica. Nuestros datos indicaron que el consumo de oxígeno de las mitocondrias aisladas de células crecidas a pH 3.7 es 20% más alto que el consumo de oxígeno de las mitocondrias aisladas de células crecidas a pH 6.0, independientemente del substrato utilizado.

ARTÍCULO III

The typically mitochondrial DNA-encoded ATP6 subunit of the F_1F_0 ATPase is encoded by a nuclear gene in *Chlamydomonas reinhardtii*

Funes S, Davidson E, Claros MG, van Lis R, Pérez-Martínez X, Vázquez-Acevedo M, King MP and González-Halphen D (2002)

J Biol Chem 277, 6051-6058

RESUMEN

El gen *atp6*, que codifica por la subunidad ATP6 del complejo F_1F_0 ATP sintasa, se encuentra en el genoma mitocondrial de la gran mayoría de los eucariontes. Sin embargo, el gen *atp6* está ausente en el mtDNA de algunos protistas, como el alga verde *Chlamydomonas reinhardtii*. Se obtuvieron las secuencias genómica y de traducción del gen *atp6* de *C. reinhardtii*, utilizando PCR, 5'-RACE y 3'-RACE. El gen *atp6* de *C. reinhardtii* se encuentra en el genoma nuclear y presenta características típicas de los genes nucleares: presencia de intrones, un uso de codones nuclear, y una señal de poliadenilación. Se confirmó que el producto del gen *atp6* forma parte del complejo F_1F_0 ATP sintasa. La proteína codificada por el gen *atp6* presenta una presecuencia de 107 aminoácidos. La hidrofobicidad promedio de la proteína es más baja en las regiones transmembranales de la proteína que no participan en el transporte de protones y que no son críticas en los contactos subunidad-subunidad. Nuestro trabajo reporta por primera vez una proteína mitocondrial de más de dos cruces transmembranales que participa directamente en la translocación de protones y que es codificada por un gen nuclear.

ARTÍCULO IV

Structure, organization and expression of the genes encoding mitochondrial cytochrome c_1 and the Rieske iron-sulfur protein in *Chlamydomonas reinhardtii*

Atteia, A., van Lis, R., Wetterskog, D., Gutierrez-Cirlos, E.-B., Ongay-Larios, Fránzen, L.-G. and González-Halphen, D. (2003)

Mol Gen Genomics 268, 637-644

RESUMEN

Se determinó la secuencia y organización de los genes nucleares que codifican para dos subunidades del complejo respiratorio bc_1 del alga fotosintética *Chlamydomonas reinhardtii*, la proteína de Rieske (Isp) y el citocromo c_1 (Cyc1). Las réplicas tipo Southern indicaron que los genes *Cyc1* y *Isp* se encuentran como copias únicas en el genoma de *C. reinhardtii*. En el gen *Cyc1* se encuentran seis intrones con un tamaño que va de 178 a 1134 pares de bases (pb). El gen *Isp* (1238 bp) contiene cuatro intrones pequeños de 83 hasta 167 pb. En ambos genes, los sitios de corte de los intrones coinciden con las secuencias consenso del sitio de corte en los organismos eucariontes: 5'-GT/AG-3'. También se encontraron secuencias internas conservadas en uno de los intrones del gen *Cyc1*. Los niveles de expresión de los genes *Isp* y *Cyc1* son comparables entre la sepa silvestre y la sepa mutante *dum*-1, la cual presenta una deleción en el gen *cob* que codifica al citocromo *b* mitocondrial. Sin embargo, ni el citocromo c_1, ni las proteínas estructurales I and II, se acumulan en las membranas de la cepa mutante. Estos datos muestran que en el alga verde *C. reinhardtii*, las subunidades del complejo de citocromos bc_1 no se pueden ensamblar en las membranas mitocondriales en ausencia del citocromo *b*.

ARTÍCULO V

Bifunctional aldehyde/alcohol dehydrogenase (ADHE) in chlorophyte algal mitochondria

Ariane Atteia, Robert van Lis, Guillermo Mendoza-Hernández, Katrin Henze, William Martin, Hector Riveros-Rosas and Diego González-Halphen

RESUMEN

Los perfiles proteicos de mitocondrias aisladas del alga heterotrófica *Polytomella* crecida en etanol a pH 6.0 y 3.7 fueron analizados en geles azules nativos y en geles desnaturalizantes de poliacrilamida con SDS. El pH del medio de cultivo afectó los niveles de acumulación de los complejos mitocondriales que participan en la fosforilación oxidativa. Los niveles de una proteína abundante y soluble, de 85 kDa y de su correspondiente RNA mensajero, fueron más altos a pH 6.0 que a pH 3.7. La obtención de secuencias del extremo N-terminal y de péptidos internos de la proteína mitocondrial de 85 kDa, permitieron obtener y secuenciar el cDNA correspondiente. La secuencia deducida permitió identificar a la proteína como una aldehído/alcohol deshidrogenasa bifuncional (ADHE). Esta ADHE presenta altas similitudes con las ADHEs de eubacterias y de protistas que carecen de mitocondrias. La proteína esta codificada como un precursor que contiene una presecuencia de 27 aminoácidos que presenta características típicas de las presecuencias mitocondriales. Un gen que codifica para un homólogo de la ADHE también fue identificado en el genoma del alga *Chlamydomonas reinhardtii*, una alga fotosintética cercana a *Polytomella*. La ausencia de genes ADHE en arqueobacterias sugiere un origen eubacteriano para la enzima de las algas clorofíceas. La ADHE has sido hasta la fecha, una proteína característica de bacterias y de eucariotes anaeróbicos. La ADHE es una proteína esencial del metabolismo energético en los protistas sin mitocondrias como *Giardia intestinalis* y *Entamoeba histolytica*. Su abundancia y su alta expresión sugieren que la ADHE juega un papel importante en el metabolismo mitocondrial del alga *Polytomella*, al menos en las condiciones estudiadas. La presencia de una ADHE en un organismo aeróbico y su expresión paralela a la de los principales complejos respiratorios es inesperada e inconsistente con la idea de que los eucariontes adquirieron el gen adhE como una adaptación a la vida anaeróbica.

Sometido a Journal of Biological Chemistry

ARTÍCULO VI : REVISIÓN

Redox-mediated light regulation of mitochondrial function and biogenesis in plants and green algae

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RESUMEN

En las celúlas fotosinteticas, la respiración mitocondrial juega un papel importante no solamente en la oscuridad, sino también en la luz. Recientemente, se han logrado avances importantes en el entendimiento del papel de las mitocondrias en la luz. Es probable que los efectos de la luz lleguen a los distintos compartimentos celulares, como la mitocondria o el núcleo, por medio de varios mensajes redox originados en el cloroplasto. En esta revisión, de acuerdo con los datos experimentales disponibles, analizamos la posibilidad de que la actividad y la biogénesis mitocondrial estén reguladas por dichos mensajes redox.

Sometido a Photosynthesis Research

RESULTADOS ADICIONALES (NO PUBLICADOS)

Figura A: SDS-PAGE de segunda dimensión donde se comparan los complejos V de las algas. Las mitocondrias puras de *C. reinhardtii* y de *Polytomella* sp. se extrajeron con cloroformo.

Figura B: Alineamiento múltiple de secuencias de COXVIb de varios organismos.

Figura C: Alineamiento múltiple de las secuencias de COXVb de varios organismos.

Figura D: Alineamientos múltiples de la secuencia de COXVIa de *C. reinhardtii* con las secuencias de COXVa o COXVIa de varios organismos.

Figura E: Geles de glicina-SDS-PAGE de dos dimensiones de las proteínas mitocondriales de *C. reinhardtii*, obtenidos de células crecidas en medio de cultivo en presencia y en ausencia de cobre.

Figura F : Gel de glicina-SDS-PAGE de sos dimensiones comparando los complejos III de *C. reinhardtii* y *Polytomella* sp.

Figura G : Comparación de los complejos respiratorios y especialmente del compejo IV de *C. reinhardtii* y *Polytomella* sp. Se muestra también la separación de las subunidades COXIIa y COXIIb de *Polytomella* sp.

Figura H : Análisis del contenido en citocromos en las mitocondrias de *C. reinhardtii* y *Polytomella* sp.

Figura I : Curvas de crecimiento de células de *C. reinhardtii*, cepa CW15, en la luz y en la obscuridad.

Figura J : Fotos de microscopía electrónica de transmisión de células de la cepa CW15 de *C. reinhardtii*, crecidas en la luz y en la obscuridad en los medios TAP y H3.

Figura K : Análisis de BN-PAGE en segunda dimensión de mitocondrias puras de *C. reinhardtii*, aisladas a partir de células crecidas en el medio H3 en la luz y en la obscuridad.

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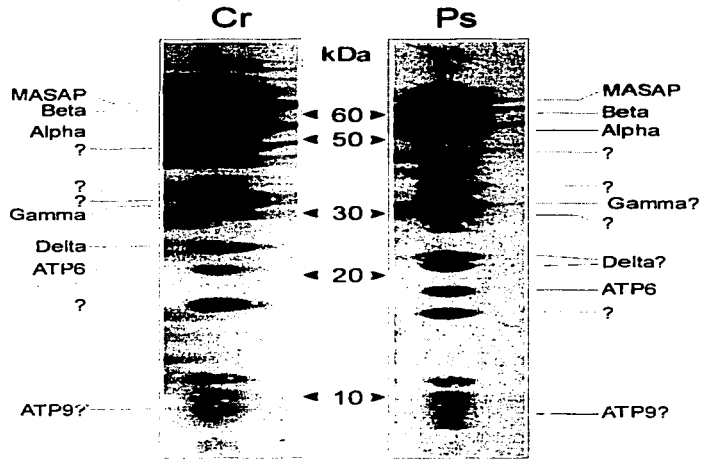

Figura A1. . Gel de glicina-SDS-PAGE (15% de acrilamida) de segunda dimensión de los complejos V de *C. reinhardtii* (Cr) y de *Polytomella* sp (Ps). Mitocondrias aisladas y solubilizadas fueron aplicadas en un gel BN-PAGE con un gradiente de poliacrilamida del 5 al 12%. Las bandas del complejo V de la primera dimensión fueron cortadas y utilizadas para la segunda dimensión. Se observa que la MASAP de *Polytomella* sp. se separa de la subunidad beta, mientras en *C. reinhardtii* este no es el caso. Parece que la MASAP de *Polytomella* sp. tiene una masa molecular más grande que la de *C. reinhardtii*. En el alga verde, para poder separar la MASAP de la subunidad beta se requirió agregar 8 M de urea en el gel. Las proteínas por abajo de la subunidad alfa y arriba o alrededor de la subunidad gama son las proteínas adicionales del complejo V todavía no identificadas. En *C. reinhardtii*, hasta 16 subunidades/proteínas pueden ser detectadas, en *Polytomella* sp. pueden observarse hasta 17 subunidades.

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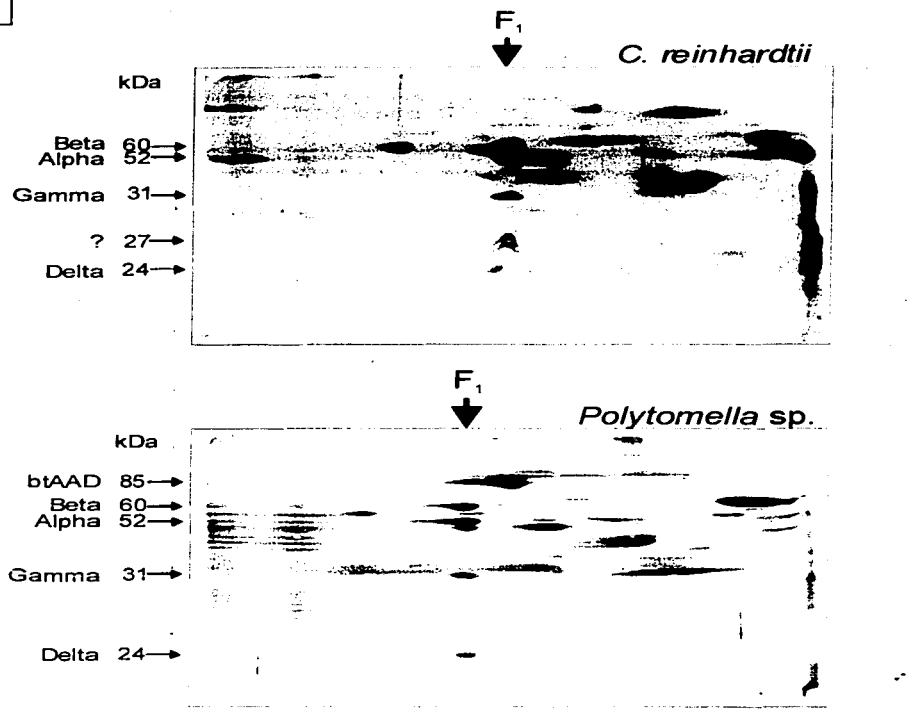


Figura A2. SDS-PAGE de segunda dimensión en presencia de urea 8 M de las proteínas extraídas con cloroformo a partir de mitocondrias puras de *C. reinhardtii* y mitocondrias de *Polytomella* sp. La extracción con cloroformo libera la fracción soluble de las mitocondrias, y también la parte soluble F_1 del complejo V. A la fase acuosa de la extracción se le adicionó n-dodecil-maltósido al 1 %, azul de Coomassie Serva Blue G 0.25 %, y se cargó en un gel azul nativo BN-PAGE. Se usó un gradiente de poliacrilamida del gel azul nativo del 5 al 12 %. La segunda dimensión se llevó a cabo en presencia de 8 M de urea para poder separar la MASAP de la subunidad beta del complejo V de *C. reinhardtii*. La MASAP y las otras proteínas no identificadas no forman parte integral de la F_1 del complejo V en las dos algas.

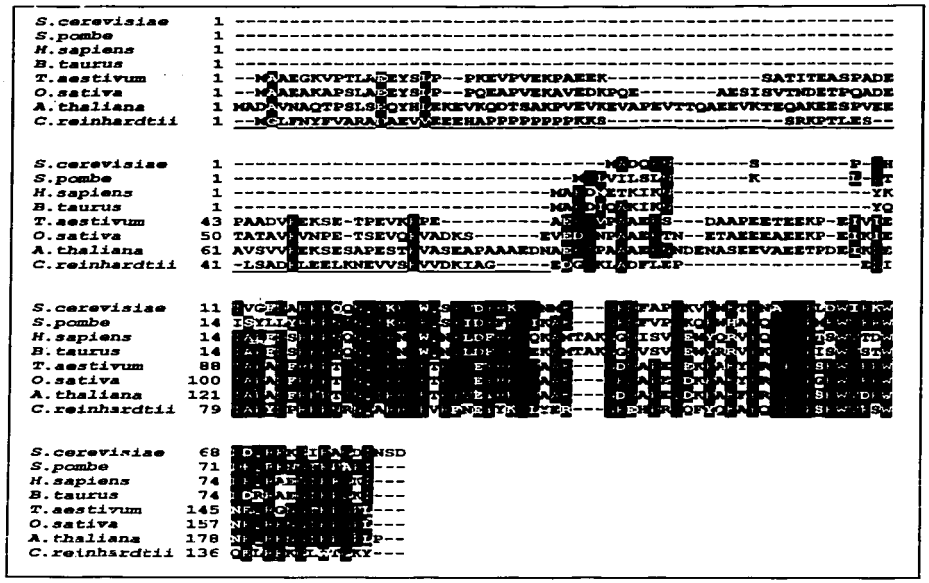
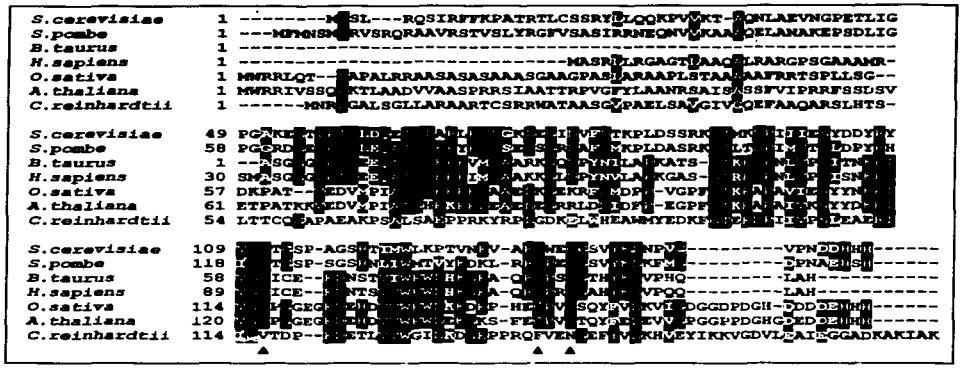


Figura B. Alineamiento múltiple de secuencias de COXVIb de varios organismos. Está subrayada la extensión que se encuentra en plantas y en *C. reinhardtii*. *S. cerevisiae*, levadura (Q01519); *S. pombe, Schizosaccharomyces pombe* (CAA21442); *H. sapiens*, humano (P14854); *B. taurus*, bovino (P00429); *T. aestivum*, trigo (AAM92706); *O. sativa*, arroz (BAB12338) *A. thaliana, Arabidopsis thaliana* (BAA87883); *C. reinhardtii, Chlamydomonas reinhardtii* (BE122218, ChlamyEST).

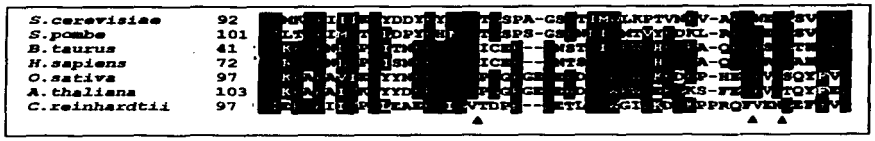
1.



		Seq1	Seq2	Seq3	Seq4	Seq5	Seq6	Seq7
Sequence 1:	*S.cerevisiae* 155 aa	100	46	34	25	27	21	13
Sequence 2:	*S.pombe* 164 aa		100	38	27	22	24	16
Sequence 3:	*B.taurus* 90 aa			100	65	37	35	17
Sequence 4:	*H.sapiens* 129 aa				100	37	27	17
Sequence 5:	*O.sativa* 169 aa					100	55	16
Sequence 6:	*A.thaliana* 176 aa						100	17
Sequence 7:	*C.reinhardtii* 117 aa							100

Figura C1. Alineamiento múltiple de las secuencias de COXVb de varios organismos (COXIV en levaduras). Las cisteínas que están involucradas en el enlace del átomo de zinc están indicadas con triángulos. En la secuencia de *C. reinhardtii*, ninguna de las tres cisteínas conservadas está presente. El porcentaje de similitud que guardan las secuencias entre sí se indican en los triángulos. *S. cerevisiae*, levadura (NP_011328); *S. pombe, Schizosaccharomyces pombe* (P79010); *H. sapiens*, humano (P10606); *B. taurus*, bovino (P00428); *O. sativa*, arroz (BAA12797); *A. thaliana, Arabidopsis thaliana* (AAM64879); *C. reinhardtii, Chlamydomonas reinhardtii* (BG851120, ChlamyEST).

2.



			Seq1	Seq2	Seq3	Seq4	Seq5	Seq6	Seq7
Sequence 1:	*S.cerevisiae*	52 aa	100	55	37	35	34	32	26
Sequence 2:	*S.pombe*	52 aa		100	41	37	30	50	32
Sequence 3:	*B.taurus*	51 aa			100	86	50	32	37
Sequence 4:	*H.sapiens*	51 aa				100	50	50	41
Sequence 5:	*O.sativa*	53 aa					100	79	34
Sequence 6:	*A.thaliana*	53 aa						100	34
Sequence 7:	*C.reinhardtii*	52 aa							100

Figura C2. Alineamiento múltiple de las secuencias de COXVb de varios organismos (COXIV en levaduras) mostrando únicamente la región central, que es la conservada. La similitud de esta parte en la secuencia de *C. reinhardtii* con las de animales es más alta que con las secuencias de plantas (vea el recuadro). El porcentaje de similitud que guardan las secuencias entre sí está indicado con triángulos. *S. cerevisiae*, levadura (NP_011328); *S. pombe, Schizosaccharomyces pombe* (P79010); *H. sapiens*, humano (P10606); *B. taurus*, bovino (P00428); *O. sativa*, arroz (BAA12797); *A. thaliana, Arabidopsis thaliana* (AAM64879); *C. reinhardtii, Chlamydomonas reinhardtii* (BG851120, ChlamyEST).

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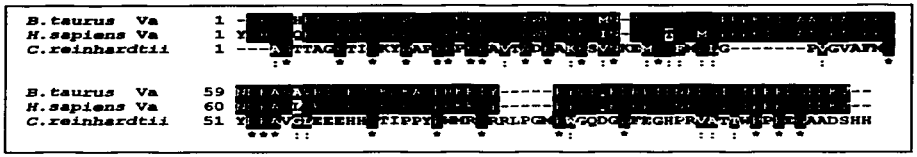

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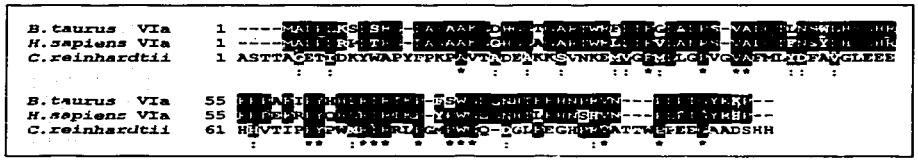
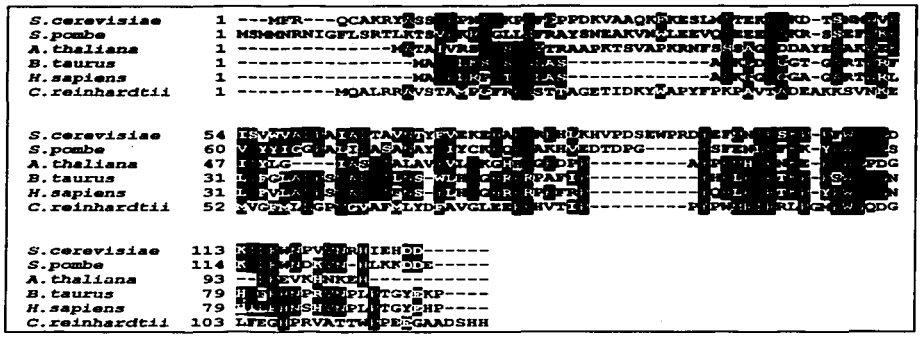


Figura D. 1) Alineamiento múltiple de la secuencia de COXVIa de *C. reinhardtii* con las secuencias de COXVa de animales. La región N-terminal de COXVIa de *C. reinhardtii* (42 aa) tiene una similitud mayor con las secuencias de COXVa de animales (31%) que con la parte C-terminal (18% sobre 66 aa). **3)** Alineamiento multiple de la secuencia de COXVIa de *C. reinhardtii* y las secuencias de COXVIa de animales. En este caso, la parte C-terminal de COXVIa de *C. reinhardtii* tiene una similitud mayor con las secuencias de COXVIa de animales (25 % en los ultimos 49 aa) que con la parte N-terminal (9% en los primeros 55 aa). *B. taurus,* bovino (COXVa, P00426; COXVIa, P07471); *H. sapiens*, humano (COXVa, P20674; COXVIa, Q02221); *C. reinhardtii, Chlamydomonas reinhardtii* (COXVIa; BG857268, ChlamyEST).

3.



4.

Figura D. 3) Alineamiento múltiple de la secuencia de COXVIa de varios organismos. La secuencia de la COXVIa parece no estar no muy conservada entre animales, plantas y levaduras. **4)** Alineamiento de COXVIa de *C. reinhardtii* con la proteína homóloga a COXIVa de *A. thaliana*. La similitud entre las dos secuencias es de 32 % en los aminoácidos 18–88 de la secuencia de *C. reinhardtii*. *S.cerevisiae*, levadura (CAA51479); *S.pombe, Schyzosacharomyces pombe* (CAA20783); *H. sapiens*, humano (Q02221); *B. taurus*, bovino (P07471); *A. thaliana, Arabidopsis thaliana* (AAK00391); *C. reinhardtiii, Chlamydomonas reinhardtii* (BG857268, ChlamyEST).

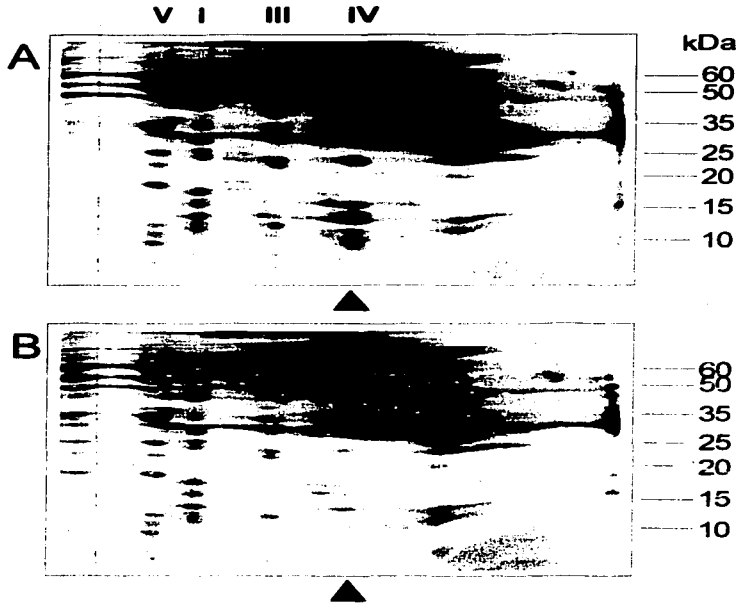


Figura E. Geles de glicina-SDS-PAGE (15 % de acrilamida) de segunda dimensión con proteínas mitocondriales de *C. reinhardtii*, obtenidas a partir de células crecidas en medio de cultivo (TAP) en la presencia y ausencia de cobre. La posición original de los complejos en la primera dimensión está indicada por los números romanos correspondientes. Los triángulos abajo del gel también indican la posición del complejo IV. A: Proteínas mitocondriales (350 µg) de células de *C. reinhardtii* crecidas en presencia de cobre en el medio de cultivo. B: Proteínas mitocondriales (350 µg) de células de *C. reinhardtii* crecidas en ausencia de cobre en el medio de cultivo. La disminución en la acumulación del complejo IV en el proteoma mitocondrial es notable cuando las células crecen sin cobre.

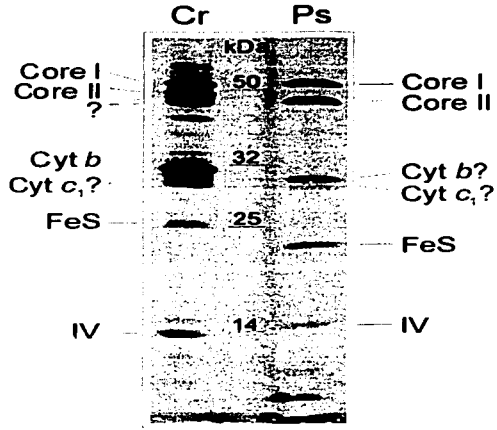
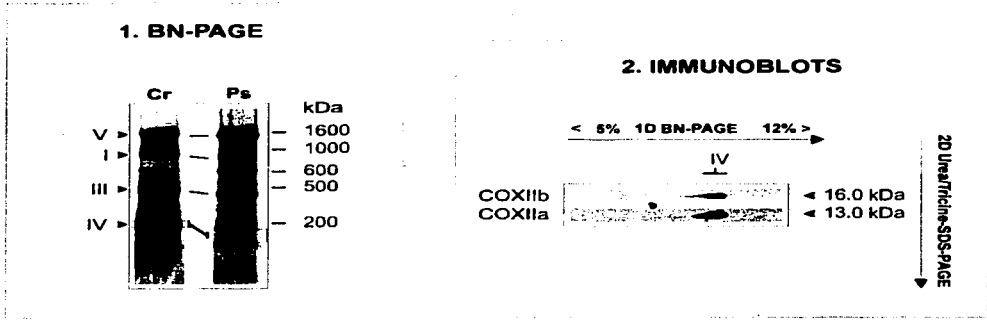


Figura F. Gel de glicina-SDS-PAGE (12% de acrilamida) de segunda dimensión con los complejos III de *C. reinhardtii* (Cr) y *Polytomella* sp. (Ps). Mitocondrias aisladas de ambas algas fueron aplicadas en geles de BN-PAGE con un gradiente de poliacrilamida del 5 al 12%. Las bandas del complejo III de la primera dimensión fueron cortadas y utilizadas para la segunda dimensión. Los subunidades del complejo III de las dos algas se separaron en el mismo gel, por lo que ambos perfiles electroforéticos son comparables. En geles de tricina-SDS-PAGE, normalmente no se observan las bandas adicionales del complejo III de *C. reinhardtii*, o solamente se observa una banda adicional que no se separa adecuadamente de la banda que coresponde a la subunidad II. Los geles de glicina-SDS-PAGE separan mejor a las subunidades con masas moleculares superiores a los 25 kDa. El gel fue teñido con plata.



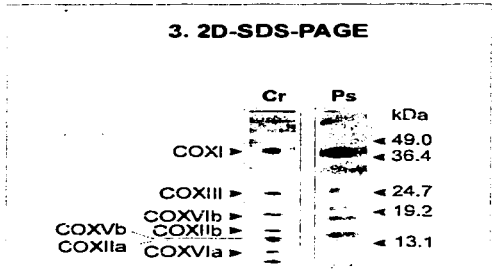


Figura G. Comparación de los complejos respiratorios y especialmente del complejo IV de *C. reinhardtii* (Cr) y *Polytomella* sp. (Ps). También se muestra la separación de las subunidades COXIIa y COXIIb de *Polytomella* sp. **1)** Mitocondrias puras y solubilizadas fueron aplicadas en BN-PAGE (gradiente de 5-12% de acrilamida), para mostrar las diferencias entre los complejos. La banda en *Polytomella* sp. que parece corresponder a la banda de complejo IV en *C. reinhardtii* es en realidad la aldehído/alcohol deshidrogenasa de tipo bacteriana (ADHE), mientras el complejo IV en este alga verde forma dos o más bandas delgadas de una masa molecular más pequeña que el complejo IV del alga verde (no visible en la figura). **2)** Separación de las subunidades COX2a y COX2b del complejo IV de *Polytomella*. Las dos proteínas fueron detectadas por hibridación tipo Western usando anticuerpos convencionales dirigidos contra péptidos sintéticos basados en las secuencias de las dos subunidades de *Polytomella* sp. Las dos subunidades se separaron en geles de tricina-SDS-PAGE (15% de acrilamida) en la presencia de 8M urea. **3)** Perfiles de las subunidades de los complejos IV de las dos algas en la segunda dimensión de BN-PAGE en geles de tricina-SDS-PAGE (15% de acrilamida). Se puede ver que en el alga incolora, las bandas forman un barrido que indica la difusión del complejo IV en la primera dimensión. Dicho barrido puede indicar múltiples formas del complejo.

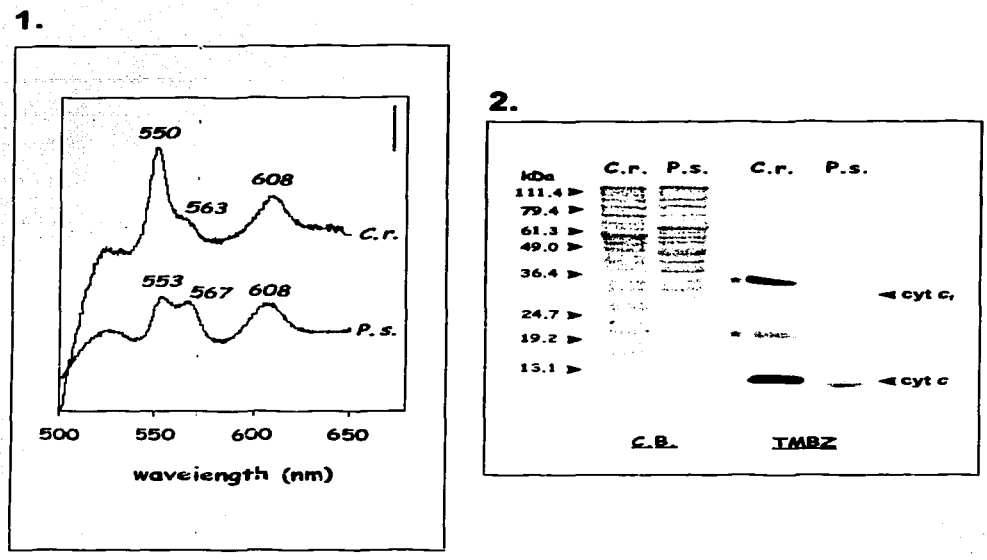


Figura H. Análisis del contenido en citocromos en las mitocondrias de *C. reinhardtii* y *Polytomella* sp. por espectrofotometría y por geles de tricina-SDS-PAGE (15% de acrilamida). **1)** Espectros diferenciales (reducidos con ditionita- *menos* oxidados por aire) de mitocondrias aisladas de *C. reinhardtii* y *Polytomella* sp. Las mitocodrias fueron aisladas de células de *C.* reinhardti crecidas en el medio TAP (***C.r.***) y de células de *Polytomella* sp. crecidas en acetato a pH 6.0 (***P.s.***). Las mitocondrias se resuspendieron en fosfato de potasio 50 mM (pH 7.4) a una concentración de proteínas de 3.0 mg/ml para *C. reinhardtii* y de 6.0 mg/ml para *Polytomella* sp. La reducción de los citocromos se logró con la adición de un poco de ditionita sólida. La barra representa 0.02 unidades de absorbencia. **2)** Geles de tricina-SDS-PAGE (15% de acrilamida) de células enteras (100 µg de proteínas) de *C. reinhardtii* (***C.r.***) y *Polytomella* sp. (***P.s.***). Los geles fueron teñidos con azul de Coomassie (C.B.) o con tetrametilbenzidina (Thomas y cols., 1978) para visualizar la actividad de peroxidasa de los grupos hemo. Los asteriscos (*) indican la posición de los dos grupos hemo que pertencen al cloroplasto en *C. reinhardtii*, el citocromo *f* (38kDa) y el citocromo b_6 (20kDa).

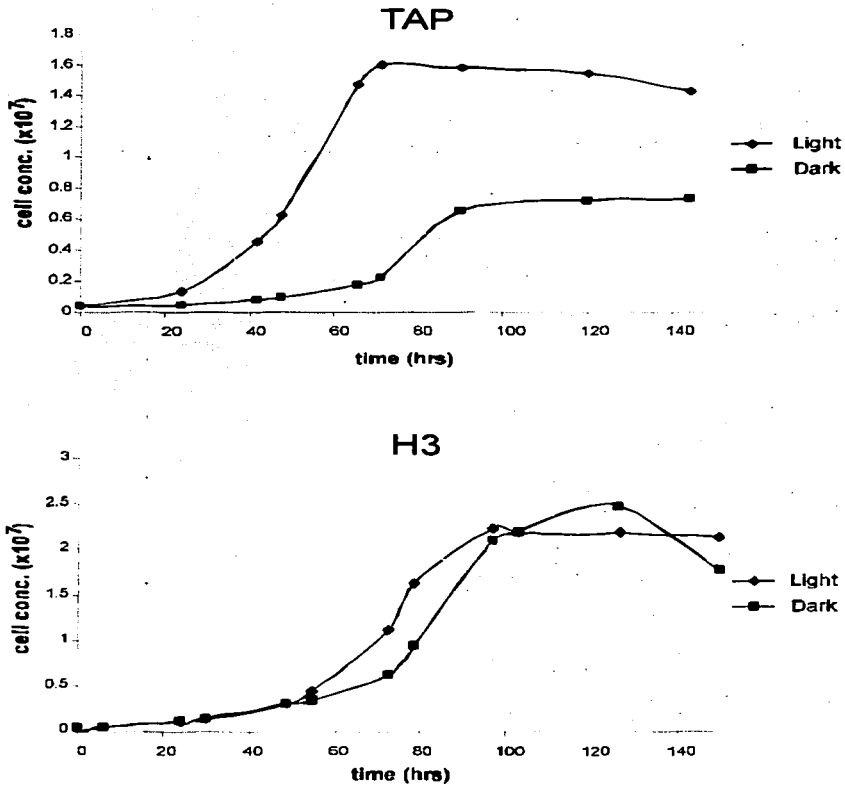


Figura I. Curvas de crecimiento de células de *C. reinhardtii*, cepa CW15 en luz y en la obscuridad en el medio TAP y H3. Para medir la concentración de las células, el cultivo fue diluído 5-20x en una solución de Lugol en 0.85 % NaCl o en una solución de 0.85 % NaCl, pH 3.0 (utilizando 10 mM acido tartárico como amortiguador). De esta dilución de células, se aplicaron 10 µl a un contador de células de Neubauer, haciéndose tres conteos independientes.

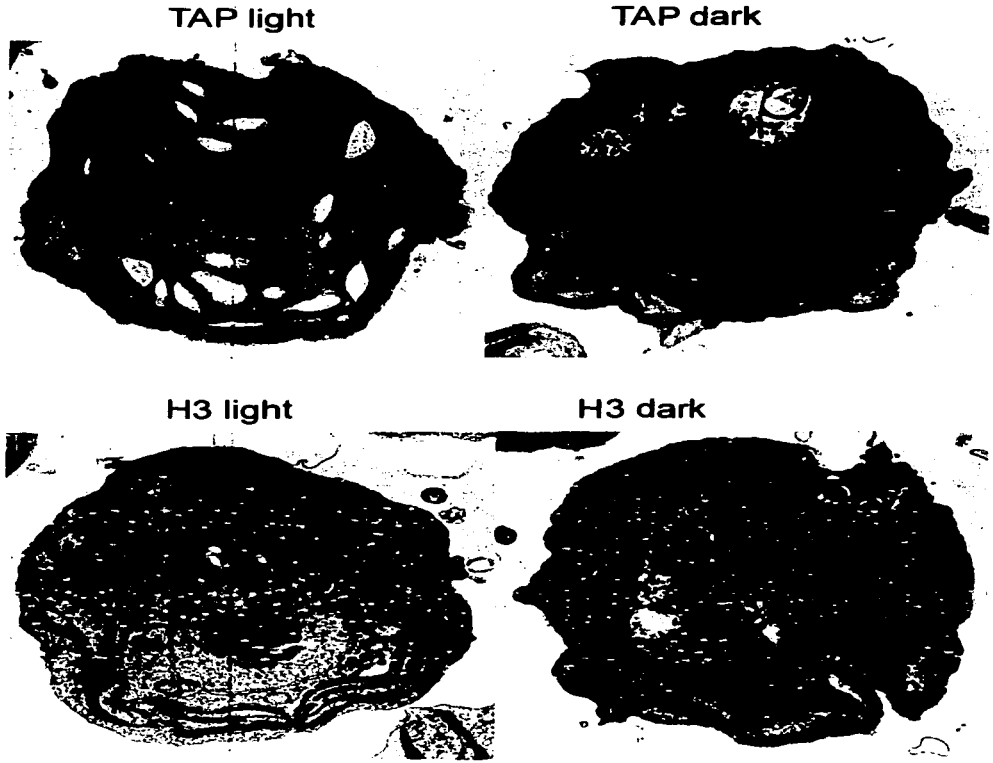


Figura J. Micrografías electrónicas de transmisión de células de la cepa CW15 de *C. reinhardtii*, crecidas en la luz y en la obscuridad en los medios TAP y H3. Las células fueron fijadas durante la noche en 4% de paraformaldehído, y las imágenes fueron tomadas de cortes a una amplificación de 5000x. El desarollo del cloroplasto, la acumulación del almidón y la cantidad y tamaño de las mitocondrias dependen de la luz y la concentración de acetato. Las imágenes fueron obtenidas por el Sr. Jorge Sepúlveda en la Unidad de Microscopía Electrónica del IFC.

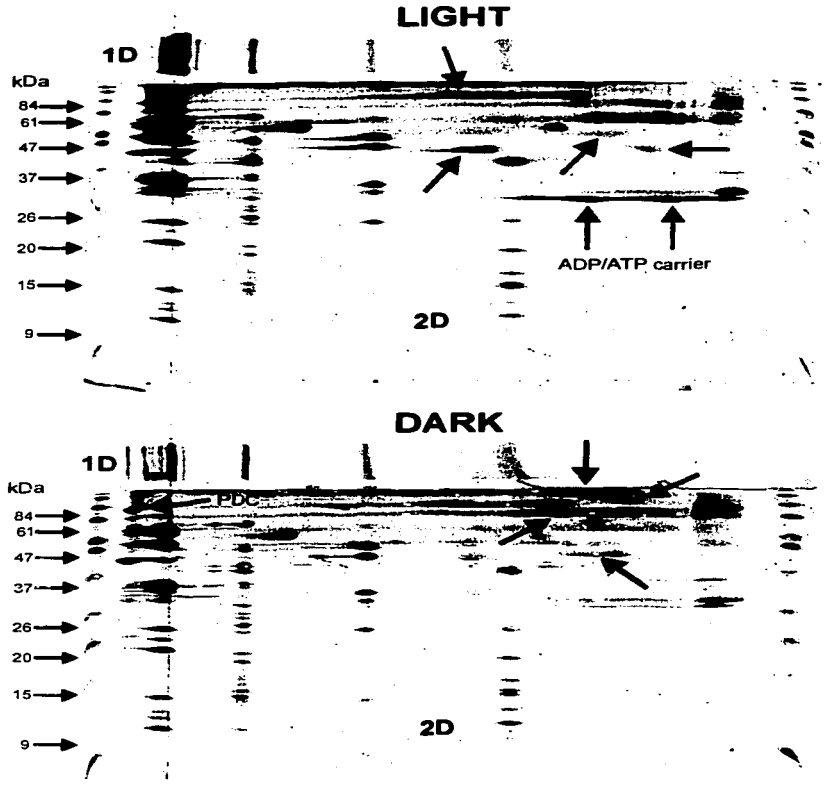


Figura K. Separación de los complejos respiratorios en geles azules nativos BN-PAGE y la resolución de las subunidades de los complejos y de otras proteínas en geles de tricina-SDS-PAGE (15 % de acrilamida) de segunda dimensión. Se cargaron en los geles mitocondrias puras solubilizadas aisladas de células crecidas en la luz y la obscuridad, en el medio H3 (50 mM acetato). Las flechas indican algunas diferencias en la biogénesis mitocondrial entre las dos condiciones experimentales. ADP/ATP, transportador de ADP/ATP; PDC, complejo de la piruvato deshidrogenasa.

DISCUSIÓN

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Aislamiento de las mitocondrias de las algas *C. reinhardtii* y *Polytomella* sp. y su análisis en BN-PAGE

En el pasado, se han realizado pocos estudios bioquímicos de las mitocondrias de algas clorofíceas, debido a que entre otras cosas, es difícil aislar organelos intactos en cantidades suficientes. El protocolo para aislar las mitocondrias de las algas *C. reinhardtii* y *Polytomella* sp. que se utilizó en este trabajo consiste en la ruptura de las células con perlas de vidrio, seguida por dos centrifugaciones y para el caso del alga fotosintética, un gradiente de densidad en Percoll (Figura 12, Introducción) (Ericksson y cols., 1995). Los intentos de purificar las mitocondrias del alga incolora en gradientes de Percoll dieron resultados negativos: las mitocondrias se quedaron arriba del gradiente. Esa diferencia en el comportamiento de las mitocondrias de las dos algas en gradientes de Percoll sugiere diferencias en la composición de los organelos, quizá en el contenido de ácidos grasos y/o de proteínas.

Las mitocondrias de *C. reinhardtii* analizadas en este trabajo fueron aisladas principalmente de una cepa mutante que carece de pared celular (cepa 84CW15). Varios factores mostraron ser importantes en el protocolo de aislamiento. Uno de los más importantes es la ruptura con perlas de vidrio. La ruptura de las células sacudiendo manualmente la suspensión celular en presencia de las perlas, resultó siempre más eficiente que agitando con un vortex. Sin embargo, resulta más difícil controlar la ruptura de las células sacudiendo la suspensión con la mano: se corre el riesgo de romperlas demasiado y de aumentar así la contaminación por material fotosintético, ya que las membranas de los tilacoides se pegan a las mitocondrias y/o forman una red que atrapa a las mitocondrias. Si esto sucede, la mayoría de las mitocondrias contaminadas no entran al gradiente, perdiéndose hasta un 90 % del material total. De un litro de medio de cultivo mixotrófico (células crecidas en luz y en presencia de acetato).se obtuvó en promedio de 2 a 3 mg de proteínas mitocondriales. Obviamente, los bajos rendimientos en las preparaciones mitocondriales de *C. reinhardtii* afectan la investigación bioquímica de este organelo.

La pared celular de las células silvestres de *C. reinhardtii* es demasiado resistente como para romperse con las perlas de vidrio. Para debilitar la pared celular, se utilizó un tratamiento con el detergente bromuro de N-cetil-trimetil amonio (CTAB) (Artículo I). Antes de romper las células con las perlas de vidrio, se lavaron las células para eliminar el detergente.

Otro método para romper las células de *C. reinhardtii* (sin o con pared celular) implica un aparato llamado BioNeb nebulizador (Glas-Col, Terre-Haute, IN) que somete a las células a un flujo de nitrógeno y las rompe al impactarlas contra una superficie. La presión del gas determina la fuerza aplicada para romper las células, y dependiendo del organismo y probablemente de la

cepa, esa presión tiene que ajustarse por ensayo y error. Con el nebulizador, la pared celular se desintegra pero el contenido intracelular se mantiene intacto, por lo que se puede utilizar para aislar diversos organelos.

Las mitocondrias aisladas de *C. reinhardtii* y *Polytomella* sp. fueron solubilizadas con el detergente *n*-dodecil-maltósido y después analizadas en geles azules nativos. Como controles y marcadores de masa molecular, se corrieron mitocondrias de bovino y de papa. Las mitocondrias de estos organismos han sido previamente caracterizadas en BN-PAGE, sin embargo, las del alga verde fueron más difíciles de resolver en la primera dimensión nativa. Los problemas con las mitocondrias del alga posiblemente se deben a su distinto contenido en ácidos grasos y/o proteínas (revisado por Daum, 1985), pero también a la cantidad importante de proteínas que se deben aplicar a dichos geles para poder ver las bandas que corresponden a los complejos respiratorios. En general, las mitocondrias en los organismos fotosintéticos contienen un mayor número de componentes (véase la Introducción), de tal manera que para ver con la misma intensidad las bandas correspondientes a los complejos respiratorios, es necesario cargar en estos geles el doble de proteína mitocondrial de *C. reinhardtii* que de bovino o de papa.

Una buena resolución de los complejos mitocondriales del alga verde sigue siendo un asunto que muestra una gran variabilidad experimental, y que también parece depender de las condiciones de crecimiento de las células.

Hallazgos novedosos de los complejos OXPHOS de *C. reinhardtii*

Utilizando principalmente el método de BN-PAGE y SDS-PAGE de dos dimensiones, logramos una descripción bioquímica general de los principales complejos respiratorios y de varias otras proteínas mitocondriales de *C. reinhardtii*. Gracias a la disponibilidad de la secuencia del genoma nuclear del alga y la base de las secuencias etiquetadas y expresadas (del inglés, 'Expressed Sequence Tags') (ChlamyEST), se logró identificar un número substancial de subunidades de los complejos proteicos mitocondriales.

El carácter atípico del complejo V

Las inmunorréplicas tipo Western y ensayos de actividad enzimática revelaron que el complejo V de *C. reinhardtii* migra en BN-PAGE como una sola forma oligomérica, que exhibe una masa molecular aparente de al menos 1600-kDa (Artículo I). Ese dato contrasta significamente con lo que se ha observado para el complejo V de diferentes organismos, como

mamíferos, levaduras o plantas, solubilizado con el mismo detergente (*n*-dodecil-maltósido) (Schägger y von Jagow, 1991; Jänsch y cols., 1996; Schägger y Pfeiffer, 2000). De hecho, en prácticamente todos los organismos, el complejo V migra en geles nativos en múltiples formas, siendo las formas mayoritarias el monómero F_1F_0 de 500-600 kDa y el subcomplejo F_1 (350-400-kDa). Para observar en geles azules nativos el dímero del complejo V de *Saccharomyces cerevisiae* o de bovino, la solubilización de las mitocondrias se tiene que hacer con bajas concentraciones de un detergente suave, como Tritón-X100 o digitonina. Esos dímeros representan solamente una fracción del complejo V total en geles azules (Arnold y cols., 1998; Schägger y Pfeiffer, 2000). Por eso, la asociación dímérica del complejo V en el alga verde parece ser mucho más fuerte que en los otros organismos estudiados hasta la fecha. Se sabe que la asociación del complejo V en dímeros juega un papel importante en el control de la biogénesis de la membrana interna mitocondrial, en particular en la formación de las crestas mitocondriales (Paumard y cols., 2002).

La banda que corresponde al complejo V de *C. reinhardtii* en geles nativos fue analizada en geles desnaturalizantes de segunda dimensión (Figura A1, resultados adicionales). De las más de 13 proteínas separadas en esta segunda dimensión, seis fueron identificadas como subunidades genuinas del complejo V. Esas proteínas son las subunidades β (60-kDa); α (52-kDa); γ (31-kDa); δ (24-kDa); ATP6 (21-kDa) y ATP9 (7-kDa). Las otras proteínas presentes en la banda del complejo V, en particular la proteína de 60.5 kDa denominada MASAP (Mitochondrial ATP Synthase Associated Protein) (Artículo I) y las proteínas que tienen un peso molecular entre 50 y 30 kDa (45, 38 y 35-kDa), no pudieron ser identificadas. La determinación de sus secuencias amino-terminales permitió la búsqueda de sus genes en la base de datos ChlamyEST. Ninguna de las secuencias de aminoácidos obtenidas (Apéndice I) presenta similitudes con las subunidades de los complejos V mitocondriales o con otras proteínas en los bancos de datos disponibles a la fecha.

Es interesante hacer notar que la MASAP y la proteína de 45-kDa fueron reportadas en otras preparaciones enriquecidas en el complejo V de *C. reinhardtii*. Por ejemplo, una preparación obtenida a partir de membranas totales del alga después de la separación de los complejos proteícos en gradientes de sacarosa (Atteia, 1994). En esos gradientes, el complejo V sedimenta entre los complejos I y III, lo que podría significar que este complejo está presente en su forma monomérica. La disociación del dímero del complejo V podría deberse a las altas concentraciones de detergente y de sales utilizadas para solubilizar a las proteínas membranales (5% Tritón X100 y 0.5 M de fosfato de potasio). Las proteínas de 45, 38 y 35-kDa también fueron identificadas por su secuencia amino-terminal en una fracción de complejo V solubilizado en la presencia de dodecil-maltósido y purificadas en una columna de intercambio aniónico (Nurani y Franzén, 1996). Parece entonces que la MASAP y las proteínas de 45, 38 y 35 kDa están

estrechamente asociadas con el complejo V. Su participación en la estructura y función del complejo V quedan por ser explorados.

La parte soluble del complejo V de *C. reinhardtii*, obtenida a partir de mitocondrias extraídas con cloroformo, fue analizada en BN-PAGE. El perfil de segunda dimensión mostró la presencia de las subunidades α, β, γ y δ, que son constituyentes típicos de la F_1 (Figura A2, resultados adicionales). Ni la MASAP ni las proteínas adicionales se encontraron asociadas con la F_1. Posiblemente, estas proteínas forman parte del sector F_0 membranal, o bien, su asociación con el sector F_1 no es lo suficientemente fuerte.

También es importante recordar que las subunidades α y β del complejo V en *C. reinhardtii* son más grandes que las subunidades correspondientes de plantas, mamíferos y levaduras (Franzén y Falk, 1992; Nurani y Franzén, 1996). La subunidad α presenta en su extremo amino-terminal una extensión de 20 aminoácidos, mientras que la subunidad β muestra a su extremo carboxilo-terminal una extensión muy hidrofílica de 60 aminoácidos. El papel de las extensiones en las subunidades α y β no ha sido investigado hasta la fecha. La MASAP parece estar presente en el complejo V en la misma estequiometría que las subunidades α y β (Artículo I, Figura A1 de los resultados adicionales). Esto podría indicar que la MASAP interactúa con estas dos subunidades, por medio de sus extensiones; esta supuesta interacción no resistiría la disociación inducida por el cloroformo. El carácter dimerico del complejo V del alga verde es posiblemente una consecuencia de la interacción de la MASAP entre sí o con otras proteínas desconocidas.

Uno se pregunta porqué una dimerización tan estable es necesaria para la complejo V del alga *C. reinhardtii*, para que sirve exactamente y cual es el papel de las subunidades adicionales asociadas con el complejo V en BN-PAGE. No se sabe si la dimerización del complejo V es tan fuerte *in vivo* como *in vitro* (BN-PAGE). Se requerirán estudios bioquímicos más avanzados para determinar la actividad, la regulación, la estabilidad y la estructura de este complejo. El complejo V en las algas clorofíceas representa un reto para el futuro. Sin duda, será interesante tener más información sobre la función y estructura del complejo V en relación con el ambiente metabólico.

Características singulares de algunas de las subunidades y de la estabilidad del complejo IV de C. reinhardtii

Como se mencionó en la introducción, varios de los genes que típicamente se encuentran localizados en el genoma mitocondrial, no están presentes en el genoma mitocondrial de las algas unicelulares *C. reinhardtii* y *Polytomella* sp.; tal es el caso de los genes *nad*4L, *cox*2, *cox*3, *atp*6 y *atp*8. Dichos genes se encontraron en el DNA nuclear de estas algas (Pérez-Martínez y cols., 2000; 2001; Funes, 2002; Artículo III).

El hecho de que la subunidad COXII, normalmente codificada por un solo gen mitocondrial (el gen *cox*2), esté codificada en *C. reinhardtii* por dos genes nucleares (*cox*2a y *cox*2b) (Pérez-Martínez y cols., 2001) fue un resultado inesperado. En SDS-PAGE, la subunidad COXII de bovino corre como una proteína de masa molecular aparente de 26 kDa (Steffens y Buse, 1979) mientras que en el perfil de las subunidades del complejo IV de *C. reinhardtii* no se puede distinguir una banda dentro de los mismos límites de masas moleculares (Artículo I). Los datos moleculares reportados anteriormente acerca del gen *cox*2 fragmentado en *C. reinhardtii* fueron completados en este trabajo con la caracterización de las proteínas COXIIa (14-kDa) y COXIIb (16-kDa), al identificar estas subunidades por sus secuencias de aminoácidos y por un análisis tipo Western (Artículo I). La resolución de la subunidad COXII en dos polipéptidos distintos en geles desnaturalizantes indica que el enlace entre las subunidades COXIIa y COXIIb es susceptible a la acción del SDS. Por lo tanto, se infiere que estas proteínas de la membrana interna mitocondrial no están unidas de manera covalente entre sí. La secuencia amino terminal de la subunidad COXIIa madura de *C. reinhardtii* coincide con el sitio de escisión predicho con base en la secuencia de aminoácidos deducida a partir del gen nuclear *cox*2a. En cambio, la degradación de Edman de la subunidad COXIIb generó una secuencia que se encuentra a una distancia de 96 aminoácidos del extremo amino-terminal predicho por la secuencia del gen (Pérez-Martínez y cols., 2001). Esto parece indicar que el extremo amino-terminal de la subunidad COXIIb en el alga está bloqueada (formilada o acetilada) y que ocurre un tipo de degradación específica de la proteína durante el proceso de la degradación de Edman.

El significado de la organización del gen *cox*2 no se conoce en este momento, pero parece ser una característica de varias algas clorofíceas. Será interesante identificar que otras algas carecen del gen *cox2* en su genoma mitocondrial. Establecer los nexos entre esos organismos podría ayudar en entender el significado metabólico o evolutivo del gen *cox*2 fragmentado.

No solamente las subunidades COXII, sino otras subunidades del complejo IV de *C. reinhardtii* identificadas en ese trabajo exhiben características singulares; tal es el caso de las subunidades COXVIb, COXVa y COXVIa, codificadas por genes nucleares. Estas subunidades

no son de membrana y están principalmente involucradas en la estabilidad del las subunidades catalíticas y en el ensamble del complejo IV (Burke y Poyton, 1998). Un átomo de zinc presente en la subunidad COXVb también parece tener una función en la estabilidad del complejo pero es dispensable para la actividad (Pan y cols., 1991). Se sabe poco acerca del origen evolutivo de las subunidades del complejo IV que son codificadas por genes nucleares (Grossman y Lomax, 1997).

Los primeros 60 aminoácidos de la proteína madura COXVIb (18-kDa), no muestran ninguna similitud con la subunidad correspondiente en animales. En los mamíferos, la subunidad COXVIb es más pequeña (10.1 kDa; Tanaka y cols., 1981), por lo que los 60 aminoácidos del extremo amino-terminal en la proteína de *C. reinhardtii* parecen formar una extensión (de carácter bastante hidrofóbico). En la subunidad COXVIb de algunas plantas (*Oryza sativa*; *Arabidopsis thaliana*; *Triticum aestivum*) se puede también observar una extensión en el extremo amino terminal que presenta una similitud moderada con la extensión presente en la subunidad de *C. reinhardtii* (Figura B, resultados adicionales). La extensión de la COXVIb parece estar presente sólo en los organismos fotosintéticos. Es posible que esta extensión juege un papel en el ensamble del complejo, o en la interacción del complejo IV con otros componentes mitocondriales, ya que el metabolismo y los mecanismos de regulación en aquellos organismos que llevan a cabo fotosíntesis son diferentes (Articluo VI).

La subunidad COXVb madura del alga verde (14-kDa) contiene aproximadamente 38 aminoácidos en el extremo amino terminal y otros 27 aminoácidos en el extremo carboxiio terminal que muestran poca o ninguna similitud con otras subunidades COXVb , ni siquiera con las secuencias de las proteínas de plantas. El alineamiento de las secuencias de las subunidades COXVb indica una baja similitud entre los extremos amino terminales o carboxi terminales de los organismos (Figura C1, resultados adicionales). La similitud entre las COXVb aumenta en la parte central de la proteína, en una región de alrededor de 52 aminoácidos. En esta región central, la COXVb de *C. reinhardtii* presenta una similitud moderada con las secuencias de animales (39% promedio) y plantas (34 %) (Figura C2, resultados adicionales).

Como se mencionó antes, se sabe que la subunidad COXVb une un átomo de zinc. Dicho átomo metálico está unido a tres cisteínas muy conservadas (Rizzuto y cols., 1991). De manera sorprendente, ninguna de las tres cisteínas está presente en la secuencia de COXVb de *C. reinhardtii*, ni siquiera cerca de las posiciones donde están las cisteínas en otras secuencias. Por lo anterior, se puede sugerir que la subunidad no contiene zinc. Se necesitará un análisis del complejo IV para ver si COXVb efectivamente no contiene zinc. Si este es el caso, el complejo IV de *C. reinhardtii* podría servir para estudios comparativos encaminados a elucidar la función del zinc en el complejo.

La subunidad COXVIa de *C. reinhardtii* (12 kDa) también presenta características atípicas: los primeros 42 aminoácidos presentan una similitud substancial con la COXVa de mamíferos (31%) y no con la de plantas (Figura D1, resultados adicionales). La parte carboxilo terminal exhibe una similitud de 25% con la secuencia de la subunidad COXVIa de aminales (Figura D2, resultados adicionales). La secuencia de la COXVIa parece no estar muy conservada entre animales, plantas y levaduras (Figura D3, resultados adicionales). Usando el programa FASTA3_T con la base de datos gbplnL de plantas (www2.igh.cnrs.fr/home.eng.html), solamente aparece una proteína desconocida de 102 aa en *Arabidopsis* (Figura D4, resultados adicionales) que tiene una similitud significativa (32%) con la COXVIa de *C. reinhardtii*. Sin embargo, la proteína de *A. thaliana* no exhibe similitud con la subunidad COXVa de *C. reinhardtii* en la sección amino terminal. El carácter aparentemente híbrido de la subunidad COXVIa/COXVa de *C. reinhardtii* también representa una pregunta interesante sobre la organización y el ensemblaje del complejo IV en el alga fotosintética y en las plantas.

La subunidad COXIV de mamíferos y levaduras es una proteína de aproximadamente 17-kDa que es necesaria para la estabilidad y actividad del complejo IV (McEwen y cols., 1986). En *C. reinhardtii*, se determinaron las secuencias amino terminales de todas las subunidades mayores de 10 kDa, y ninguna presenta similitudes con la subunidad COXIV de animales o de levaduras. Una búsqueda exhaustiva en el genoma de *C. reinhardtii* usando la secuencia de la subunidad IV de levadura y humano, tampoco indicó la presencia de un gen que codifique para la subunidad COXIV. La búsqueda de un gen homólogo a la subunidad COXIV en los genomas de plantas (*A. thaliana*, O. *sativa* y otras) tampoco resultó positiva, por lo que se puede inferir que los organismos fotosintéticos no tienen una subunidad equivalente a la subunidad COXIV de mamíferos o de levaduras.

Con base en las secuencias de las subunidades COX determinadas en este trabajo, se puede inferir que *C. reinhardtii* ha seguido su propio camino evolutivo: algunas subunidades se parecen más a las de los animales, otras se parecen más a las de plantas, y algunas más no se parecen a ninguna subunidad previamente descrita. Las subunidades desconocidas del complejo V constituyen otro ejemplo de esto. La variabilidad en las subunidades pequeñas de la citocromo oxidasa no se puede explicar en términos de la teoría del endosimbionte, ya que las subunidades como COXV a COXVIII no se encuentran en las enzimas de los procariontes. Sin embargo, es posible que el ancestro de esta alga y otras algas fue distinto del ancestro de animales y plantas. La diversidad entre las algas es enorme, mucho más grande que entre plantas (comunicación personal de Thomas Pröschold). En este sentido, no es tan sorprendente que las secuencias de *C. reinhardtii* exhiban diferencias con los animales y las plantas. La diversidad de las algas refleja la diversidad de sus habitats y condiciones ambientales, que las obliga a contar con una

gran capacidad de adaptación. Por lo tanto, no es extraño que tengan una regulación de sus complejos mitocondriales distinta a la de otros tipos de organismos. A lo largo de este trabajo se han presentado diversos ejemplos de esto.

C. reinhardtii es capaz de crecer fotosintéticamente en ausencia de cobre, a pesar de no sintetizar una proteína esencial como la plastocianina. Esto se debe a que la función de la plastocianina puede ser reemplazada por el citocromo c_6 (Wood, 1978), una proteína de 12-kDa que transporta electrones del complejo b_6f al fotosistema I como parte de CTE fotosintética. El cobre también es un componente redox del complejo IV (Tsukihara y cols., 1996). Se ha mostrado que en la ausencia de cobre, los niveles de la subunidad COXIIb del complejo IV disminuyen, mientras que los niveles de acumulación de la proteína AOX aumentan (Nakamoto, 2001). En la Figura E (resultados adicionales) se ilustra que el crecimiento de *C. reinhardtii* sin cobre causa que la acumulación del complejo IV disminuya hasta niveles prácticamente indetectables, mientras que los niveles de los complejos I, III, y V no parecen estar afectados. La disminución drástica del contenido en complejo IV confirma que el cobre es necesario para la estabilidad del complejo IV. El efecto de la ausencia de cobre constituye un ejemplo del uso de BN-PAGE para analizar la biogénesis mitocondrial, y el efecto de diversas condiciones en el medio de cultivo.

El complejo III y la actividad de la peptidasa procesadora mitocondrial (MPP)

El complejo III de *C. reinhardtii* también fue sujeto al análisis molecular y bioquímico. En el artículo IV, se reportaron las secuencias de los genes nucleares que codifican a dos subunidades del complejo III, el citocromo c_1 (*cyc*$_1$) y la proteína fierro-azufre tipo-Rieske (*Isp*). En ese mismo artículo demostramos que en una cepa mutante de *C. reinhardtii* que carece de citocromo *b* (debido a una interrupción en el gen mitocondrial *cob*), ninguna de las subunidades del complejo III (proteínas estructurales I, II, cyt c_1, ISP) se acumula en las membranas mitocondriales. Sin embargo, un análisis tipo Northern, usando sondas para detectar los mensajeros de los genes *cyc*$_1$ e *Isp*, indicó niveles equivalentes de expresión entre la cepa mutante y la cepa silvestre. Por eso se puede inferir que la falta de una subunidad clave del complejo, como el citocromo *b*, causa la instabilidad y la degradación de las otras subunidades del complejo. Este fenómeno es conocido como "Control por epistasis de la síntesis" CES (del inglés Control by Epistasy of Synthesis) y había sido reportado para los complejos del cloroplasto en el alga verde (Choquet y cols., 2001). En el párrafo anterior se mostró que la inhabilidad para sintetizar las subunidades funcionales COXI y COXIIb por la ausencia de cobre en el medio de cultivo tiene un efecto similar al CES, en este caso la inestabilidad del complejo IV.

En este trabajo también se analizaron las dos proteínas estructurales del complejo III (las subunidades I y II) del alga verde *C. reinhardtii* (Artículo I). En plantas, además de sus funciones en el transporte de electrones, el complejo III exhibe una actividad de peptidasa procesora, cortando las presecuencias de las proteínas mitocondriales codificadas en el núcleo al momento de ingresar al interior de la mitocondria (MPP)(Braun y Schmidt, 1995a). La actividad de peptidasa ha sido localizada en las proteínas estructurales: la subunidad I presenta una actividad equivalente a la subunidad beta de la MPP, y la subunidad II presenta actividad de alfa-MPP. A partir de las secuencias en la base de datos ChlamyEST, se reconstruyeron las secuencias probables de estas proteínas estructurales. Las secuencias obtenidas fueron alineadas con las de otros organismos. El alineamiento múltiple de las secuencias de la subunidad I indica que *C. reinhardtii* sí debe presentar actividad de MPP (MPP beta), ya que está presente el motivo de enlace de zinc (H-X-X-E-H), que se encuentra en todas las proteínas que poseen actividad MPP beta (Kitada y cols., 1995). Sin embargo, la subunidad II del alga verde no debería tener una actividad de MPP alfa, ya que está ausente la secuencia consenso (Braun y Schmitz, 1995b). Anteriormente se había reportado que en el hongo *Neurospora crassa*, la proteína soluble llamada PEP (peptidase enhancing protein, equivalente a la MPP alfa), presente en la matriz mitocondrial, es importante para la actividad MPP de la proteína estructural I del complejo III (Hawlitschek y cols., 1988). En el alga verde *Euglena gracilis*, la actividad de MPP se encuentra también asociada a la proteína estructural I, pero la subunidad MPP alfa es soluble (Braun y Schmitz, 1995b, Brumme y cols., 1998)

Nurani y cols. (1997) reportaron que en *C. reinhardtii*, la actividad de MPP es soluble. Esto podría resultar de la disociación de una parte de la subunidad I del complejo III, pero es posible que esta actividad soluble sea comparable con una actividad de MPP soluble que se encontró en cotiledones de soya y en hojas de espinaca, además de la actividad integral del complejo III (Szigyarto y cols., 1998). Las proteínas estructurales y las subunidades de la MPP son proteínas parecidas estructuralmente, pero su relación evolutiva no es clara todavía (Braun y Schmitz, 1995b).

Las mitocondrias en las algas unicelulares del orden Chlamydomonadales

El alga fotosintética *C. reinhardtii* y el alga incolora *Polytomella* sp. pertenecen a la familia de las Chlamydomonaceas, o según la clasificación de Pröschold y cols. (2001) al clado 'Reinhardtii'. Después de la observación de que *C. reinhardtii* y *Polytomella* sp. están estrechamente relacionadas desde el punto de vista morfológico (Pringsheim, 1955), las secuencias de los diferentes genes que codifican para las proteínas de la cadena respiratoria

mitocondrial y la caracterización bioquímica de estas proteínas han confirmado la cercanía filogenética y evolutiva de estas dos algas (Franzén y Falk, 1992; Nurani y Franzén, 1996; Atteia y cols., 1997; Pérez-Martínez y cols., 2000, 2001). La secuenciación del genoma mitocondrial de diferentes especies de *Chlamydomonas* (*C. reinhardtii, C. moewusii, C. eugametos*) (Michaelis y cols., 1990; Denovan-Wright y Lee, 1992) y de *Polytomella parva* (Fan y Lee, 2002) fue importante para el conocimiento de las mitocondrias de las Chlamydomonaceas. Lo mismo se aplica para el aislamiento y la caracterización de los genes nucleares que codifican para algunas proteínas mitocondriales. A continuación se presenta una comparación de las mitocondrias de las dos algas, basada en su mayor parte en datos bioquímicos.

El complejo V

Los estudios del complejo V aportan una indicación fuerte de la cercanía filogenética entre *C. reinhardtii* y *Polytomella*. Todas las características excepcionales mencionadas acerca del complejo V del alga verde (véanse los párrafos superiores) también se encuentran en el alga incolora. En BN-PAGE, el complejo V *Polytomella* sp. siempre se encuentra como dímero, y su masa molecular aparente es prácticamente idéntica al del complejo V de *C. reinhardtii*. El perfil de las subunidades del complejo V de *Polytomella* sp. resuelto en un gel desnaturalizante es similar, aunque no idéntico al de *C. reinhardtii* (Figura A1, resultados adicionales). No hay datos disponibles de secuencias de nucleótidos para las subunidades de *Polytomella* sp., ya que no existe un proyecto genómico para dicha alga; no obstante, las secuencias amino-terminales que se determinaron de las subunidades α y β maduras mostraron alta similitud con las de *C. reinhardtii* (Atteia y cols, 1997). Además, un anticuerpo dirigido contra la extensión C-terminal de la subunidad β de *C. reinhardtii* da una reacción cruzada con su homóloga en *Polytomella* sp., lo cual indica también la presencia de una extensión C-terminal en esta alga incolora.

Como en el caso de *C. reinhardtii*, el complejo V de *Polytomella* sp. presenta una subunidad de alta masa molecular (70-kDa) que migra arriba de las subunidades catalíticas alfa y beta (Figura A1, resultados adicionales). Muy probablemente esa proteína es equivalente a la MASAP de *C. reinhardtii*, aúnque más grande, ya que se separa completamente de la subunidad beta.

El complejo III

Se ha inferido a partir de las secuencias de las proteínas estructurales del complejo III de *C. reinhardtii* que la subunidad I tiene actividad de MPP beta y la subunidad II carece de la actividad MPP alfa. Se ha reportado anteriormente que el complejo III de *Polytomella* sp. no posee las actividad de MPP (Brumme y cols., 1998) y en este sentido, *Polytomella* sp. se parece a *C. reinhardtii*. En el trabajo de Brumme (1998) no se analizó la fracción soluble mitocondrial para detectar la actividad MPP, por lo que tanto para *Polytomella* sp. como para *C. reinhardtii*, se requerirán datos experimentales adicionales para establecer con más detalle la organización estructural de la actividad procesadora de los precursores mitocondriales en las algas unicelulares.

En BN-PAGE, el complejo III de *C. reinhardtii* tiene una masa molecular aparente de ~ 520 kD mientras que el de *Polytomella* sp. tiene una masa molecular aparente menor de aproximadamente 470 kDa (Figura F, resultados adicionales). Para mejorar la separación de las subunidades de más de 25 kDa en geles desnaturalizantes de segunda dimensión, se usaron geles de glicina SDS-PAGE (Laemmli, 1970) en lugar de los geles tricina SDS-PAGE (Schägger y von Jagow, 1987). La presencia de varias bandas adicionales en el perfil del complejo III del alga verde llama la atención. En la posición de las proteínas estructurales (alrededor de los 50-kDa del complejo III de *C. reinhardtii* se encuentran por lo menos cuatro bandas en lugar de las dos presentes en *Polytomella* sp. Además, en la posición de los citocromos, también aparecen tres bandas en el alga verde y sólo dos en el alga incolora. En el caso del complejo III de papa analizado en SDS-PAGE con glicina, se observaron varias bandas cercanas en la posición de las proteínas estructurales I y II que representan isoformas alternas de estas dos proteínas, y que no eran visibles en geles de tricina SDS-PAGE (Braun y Schmitz, 1995a). Es poco probable que las bandas adicionales en *C. reinhardtii* representen productos de proteólisis, porque los complejos III de las dos algas fueron corridos en el mismo gel. Además, los complejos III provenían de geles azules nativos frescos; en estos tipos de geles, hay poca degradación de proteínas, ya que se corren en presencia de ácido ε-aminocaproico, un inhibidor de proteasas. Existe también la posibilidad de la contaminación con otras proteínas en la primera dimensión, pero la intensidad de las bandas es bastante fuerte y reproducible. En la gran mayoría de los geles de segunda dimensión, no había proteínas contaminantes en cantidades importantes, por lo que es poco probable que las bandas adicionales en los geles de glicina SDS-PAGE sean artificios. Suponiendo que ése sea el caso, los complejos III de *C. reinhardtii* y *Polytomella* sp. exhiben diferencias que podrían manifestarse en la diferencia en movilidad electroforética en los geles azules nativos.

Anteriormente se reportaron resultados acerca del complejo III en las algas clorofíceas. Gutiérrez-Cirlos y cols. (1999) mostraron que el citocromo *b* mitocondrial de *Polytomella* sp. tiene una banda de absorbción α corrida hasta el rojo. En cambio, el citocromo *b* del alga *C. reinhardtii* tiene una banda α típica (Atteia, 1994; Antaramián y cols., 1998). La comparación de las secuencias de aminoácidos del citocromo *b* de *Polytomella* sp. y de *C. reinhardtii* reveló algunos cambios en ciertos residuos, que podrían explicar las diferencias espectroscópicas observadas (Gutiérrez-Cirlos y cols., 1998; Antaramián y cols, 1998). Estos cambios se encuentran en los aminoácidos cercanos a las histidinas que unen al hemo y en las glicinas altamente conservadas que llevan a formar una cavidad más hidrofóbica y más estrecha para los grupos hemo.

Juntos esos datos indican sorprendentes diferencias al nivel estructural y funcional entre los complejos centrales de la cadena respiratoria del alga fotosintética y de su homóloga no-fotosintética.

El complejo IV

En *C. reinhardtii*, la subunidad COXII está codificada por los genes *cox2a* y *cox2b*, cuyos productos forman juntos una subunidad madura. En *Polytomella* sp., se ha obervado lo mismo (Pérez-Martínez y cols., 2001). Ya que se conoce este fenómeno en muy pocos organismos, la partición del gen *cox2* realmente es un evento único que claramente une filogenéticamente al alga verde con su homóloga incolora. Como en el alga verde, en *Polytomella* sp. las proteínas COXIIa y COXIIb fueron identificadas en SDS-PAGE de segunda dimensión. Las proteínas fueron detectadas por hibridación tipo Western, usando anticuerpos contra los péptidos específicos del subunidades COXIIa y COXIIb de *Polytomella* sp. Las dos proteínas fueron bien separadas y muestran una masa molecular aparente de 13 kDa para COXIIa y de 16 kDa para COXIIb. La separación de las proteínas en geles se pudo obtener al agregar 8 M de urea al gel. (Figura G2, resultados adicionales). Es importante hacer notar que la adición de urea no fue necesaria para separar las subunidades COXIIa y COXIIb de *C. reinhardtii* (Artículo I). Estas diferencias en movilidad electroforética son interesantes, ya que las secuencias de las proteínas deducidas a partir de las secuencias nucleotídicas de los genes *cox2a* y *cox2b* son bastante similares en ambas algas y no predicen diferencias importantes. Por eso los datos sugieren posibles diferencias en la conformación y/o la estabilidad de las subunidades COXIIa y COXIIb entre las algas.

Otros datos experimentales indican diferencias en las propiedades del complejo IV de *C. reinhardtii* y *Polytomella* sp. en BN-PAGE. Mientras que el complejo IV del alga verde migra

como una sola banda intensa en geles azules nativos, el complejo IV de *Polytomella* sp. migra reproduciblemente como varias bandas cercanas de menor intensidad (Figura G1 y G3, resultados adicionales). Analizando la segunda dimensión, existen por lo menos dos perfiles distintos de las subunidades del complejo IV en *Polytomella* sp. en la primera dimensión: un complejo de 180 kDa y un subcomplejo de 140 kDa. La diferencia mayor entre las dos formas de complejo IV en *Polytomella* se encuentra al nivel de una subunidad de ~ 30 kDa que podría ser la subunidad COXIII. La proteólisis específica de esta subunidad del complejo IV de *Polytomella* es poco probable. Además, en todas las condiciones de crecimiento de *Polytomella* sp. analizadas hasta la fecha (acetato o etanol, pH 3.7 o 6.0), las dos formas de complejo IV se han observado en aproximadamente la misma relación de 1:1 (comunicación personal, A. Atteia).

La forma más grande del complejo IV de *Polytomella* sp. migra más en BN-PAGE que el monómero del complejo IV de *C. reinhardtii*. La diferencia observada se podría explicar por una conformación distinta de los complejos o más probablemente por la falta de una(s) subunidade(s) en el complejo IV del alga incolora. En el complejo IV de *Polytomella* sp., sólo se han detectado 8 subunidades, en lugar de las 10 presentes en *C. reinhardtii*.

Las cuestiones sobre el significado de las diferencias y las consecuencias para la función del complejo IV de las dos algas quedan por el momento sin respuestas. Primero habrá que indentificar todas las subunidades del complejo IV del alga incolora, y estudiar los complejos aislados por diferentes metódos, midiendo su actividad, titulando con inhibidores y caracterizando su masa molecular por espectrometría de masas. En el caso particular de *Polytomella* sp., habrá que estimar la estabilidad del complejo bajo diferentes condiciones (temperatura, detergentes etc.) e investigar la presencia/ausencia de la subunidad COXIII.

Espectros de las mitocondrias aisladas

En este trabajo se registraron los espectros de mitocondrias aisladas del alga verde crecida en condiciones mixotróficas (en presencia de acetato y de luz). Los espectros diferenciales de las mitocondrias aisladas de *C. reinhardtii* comparados con los de las mitocondrias aisladas de *Polytomella* sp. crecida en acetato, revelaron contenidos distintos de los citocromos tipo *a*, *b* y *c* (Figura H1, resultados adicionales). El análisis de las células enteras y de las mitocondrias aisladas en SDS-PAGE y los estudios espectroscópicos correspondientes mostraron una diferencia en el contenido en citocromo c_{550} soluble: el alga verde contiene mucho más citocromo *c* soluble que el alga incolora (Figura H2, resultados adicionales). Los complejos IV de las algas también se comportan de manera distinta en BN-PAGE (migración y diferentes

formas del complejo). Esos datos sugieren diferencias en el transporte de electrones del ubiquinol hacia el oxígeno en las dos algas.

Los análisis de las mitocondrias de las dos algas se llevaron a cabo usando células crecidas en un medio de cultivo que contiene acetato, para poder tener condiciones comparables. Sin embargo, se sabe que *Polytomella* sp. no crece bien en acetato cuando el pH del medio de cultivo está por arriba de 6.0, por lo que se creció a pH 6.0. El pH del medio estándar TAP para *C. reinhardtii* es 7.2 (Harris, 1989). Se ha mostrado que el pH de 6.0 y el acetato no constituyen las mejores condiciones de crecimiento para *Polytomella*: el crecimiento es menor y la acumulación de almidón en los amiloplastos es más alta que en las células crecidas en etanol a pH 6.0, mientras que la diferencia es aún más grande a pH 3.7 (Artículo II). A pH 3.7 y creciendo en etanol, la expresión de los complejos respiratorios es mayor mientras que el complejo V se expresa relativamente menos, y la respiración mitocondrial exhibe un nivel más alto que a pH 6.0. Estas observaciones pueden explicar en parte la diferencia en el contenido mitocondrial de los citocromos tipo *a*, *b* y *c* entre las algas: las condiciones de crecimiento en acetato a pH 6.0 son subóptimas para *Polytomella*, y causan un metabolismo mitocondrial que aparentemente resulta en una menor respiración y en menos crecimiento celular. Pareciera que hay una menor acumulación de biomasa (menor concentración de células) pero una mayor acumulación de almidón. Como ejemplo, en *C. reinhardtii* la acumulación de almidón aumenta cuando no hay suficientes cantidades de nutrientes (Libessart y cols., 1995; Hicks y cols., 2001).

Una aldehído/alcohol deshidrogenasa de tipo bacteriano en las algas clorofíceas

En las mitocondrias de *Polytomella* sp. se observó en BN-PAGE una banda de 200 kDa, que en la segunda dimensión se separó en una banda principal de 85 kDa y otras dos de 60 kDa y 37 kDa. Estas dos proteínas juntas con la banda de 85 kDa pueden formar un complejo. Se obtuvieron las secuencias de aminoácidos del extremo amino-terminal y de algunos fragmentos trípticos internos de la proteína de 85 kDa. Estas secuencias permitieron diseñar oligonucleótidos que a su vez permitieron obtener la secuencia completa del cDNA que codifica a esta proteína. La secuencia de aminoácidos deducida muestra alta similitud con las aldehído/alcohol deshidrogenasas de tipo bacteriano (ADHE en bacterias; Artículo V). La proteína de *Polytomella* sp. se sintetiza como una preproteína con una presecuencia de 27 aminoácidos que presenta las características de un péptido señal mitocondrial (Artículo V). En todas las preparaciones mitocondriales de *Polytomella* sp., analizadas en BN-PAGE, siempre se observaron cantidades importantes de ADHE. Sin embargo, la expresión de la ADHE depende del pH del medio de

cultivo, y varía en proporciones semejantes con el complejo V mitocondrial (Artículo V). Por eso se piensa que está integrada al metabolismo mitocondrial del alga.

En bacterias como *Escherichia coli*, la ADHE es una proteína bifuncional que oxida, bajo condiciones anaeróbicas, al NADH para catalizar la conversión de acetil-CoA en aldehído y luego en etanol (Goodlove y cols., 1989). La ADHE se ha descrito solamente en bacterias como *E. coli* y *Clostridium acetobutylicum* y en eucariontes amitocondriados, como *Entamoeba histolytica* y *Giardia lamblia*.

En la base de datos del genoma de *C. reinhardtii*, hemos también detectado un gen que codifica para una aldehído/alcohol deshidrogenasa de tipo bacteriano (Artículo V). Posiblemente, la ADHE de *C. reinhardtii* presenta un péptido señal que permite su importación a la matriz mitocondrial, inferido a partir de programas que predicen el destino de preproteínas en una célula. Ya que hasta la fecha la proteína no se ha identificada bioquímicamente, no se sabe la localización de la ADHE en *C. reinhardtii*.

La proteína ADHE en el proteoma mitocondrial del alga verde, si es que está presente, presenta niveles extremadamente bajos, en comparación con el proteoma mitocondrial del alga incolora. Sin embargo, la secuencia de la ADHE en *C. reinhardtii* está presente en la base de datos ChlamyEST, lo que nos indica que la proteína se expresa. A pesar de los datos sobre la ADHE en *Polytomella* sp., solamente podemos especular acerca de la función y el significado de esta enzima de origen bacteriano en las mitocondrias de las algas.

Nunca antes se había identificado una proteína de tipo ADHE en un organismo aeróbico, ya que la enzima funciona normalmente bajo condiciones anaeróbicas. Se puede decir entonces que la presencia de la ADHE en las dos algas representa un evento evolutivo muy particular, comparable con las características extraordinarias de los complejos IV y V. Lo más probable es que el gen de la ADHE se halla originado en una eubacteria, y halla sido adquirido por las algas en un evento de transferencia lateral de genes. Es posible que la transferencia del gen ADHE ocurriera en un ancestro de *C. reinhardtii* y *Polytomella*.

En *E. coli*, la presencia de oxígeno causa la degradación de la ADHE y la disminución de su expresión (Clark y Cronan, 1980; Leonardo y cols., 1983). El análisis de las secuencias de la ADHE y de otras deshidrogenasas dependiente de hierro, ha permitido proponer que una de las tres histidinas conservadas, que une a uno de los dos átomos de hierro, es la responsable de la alta sensibilidad de la enzima al oxígeno durante la oxidación catalizada por metales (MCO, Metal-Catalyzed Oxidation) (Cabiscol y cols., 1994). La ADHE en el alga incolora no tiene estas tres histidinas, mientras que el alga verde sí las contiene, por lo que es posible que la ADHE en

C. reinhardtii sea sensible al oxígeno, y no la ADHE de *Polytomella* sp. Las cantidades importantes de la proteína en *Polytomella* sp. bajo cualquier condición de crecimiento indican que en este organismo, la ADHE es estable en la presencia de oxígeno. Si la enzima en *C. reinhardtii* efectivamente tiene características similares a la de *E. coli* (y a la de otras bacterias) se puede discutir que la enzima se expresará y funcionará sólo bajo condiciones anaeróbicas, que podrían ocurrir bajo ciertas circunstancias, como la oscuridad (Klock y Kreuzberg, 1991). Para poder analizar este punto con mayor profundidad, se necesitará la confirmación de la presencia de la ADHE en la mitocondria de *C. reinhardtii*. En este momento tampoco se puede excluir la presencia de la ADHE en el citoplasma de *C. reinhardtii*. Las diferencias entre la secuencia de la ADHE de *Polytomella* sp. y *C. reinhardtii*, que posiblemente indican una distinta sensibilidad al oxígeno, pueden sugerir la adaptación de la ADHE a su función en la mitocondria de *Polytomella* sp. Para saber más acerca de la función de la ADHE en el alga verde, se requerirá de análisis tipo Northern y de mediciones de actividad de la enzima en condiciones aeróbicas y anaeróbicas. Anteriormente se ha detectado la actividad de una aldehído deshidrogenasa depediente de la CoA (ALDH) en cloroplastos y mitocondrias (Kreuzberg y cols., 1987). Esta ALDH normalmente no se encuentra en eucariontes; el único gen que codifica para una ALDH en *C. reinhardtii* es parte del gen que codifica para la ADHE. Esto indicaría la localización de la ADHE en los organelos en *C reinhardtii*. Es interesante que también se ha reportado la presencia de una actividad de liasa de ácido pirúvico-ácido fórmico (PFL; EC 2.3.1.54) en los cloroplastos y las mitocondrias de *C. reinhardtii*, exclusivamente bajo condiciones anaeróbicas (Kreuzberg y cols., 1987). La PFL es una enzima, principalmente presente en bacterias, que cataliza la formación de acetilCoA y ácido fórmico a partir de piruvato y CoA. Se ha alineado la secuencia deducida de aminoácidos de la PFL de *C. reinhardtii* (Dumont y cols., 1993; contig 20021010.7098.1, ChlamyEST) por el programa FASTA. Resultó que la PFL del alga verde se parece hasta 62 % a las de bacterias, como *Escherichia coli*, *Clostridium pasteurianum* y *Streptococcus mutans*. En *E. coli*, la ADHE juega el papel de PFL-desactivasa (Kessler y cols., 1991). Experimentos futuros habrán de determinar las condiciones requiridas para la expresión de la ADHE en *C. reinhardtii*, y si también es capaz de desactivar la PFL.

Reflexiones acerca de las mitocondrias de C. reinhardtii y Polytomella sp.

La pérdida secundaria de la fotosíntesis constituye un fenómeno muy interesante en la evolución de los organismos. Se piensa que las algas incoloras del género *Polytomella* han evolucionado a partir de un ancestro fotosintético del clado Reinhardtii (Round, 1980) lo cual puede explicar las múltiples similitudes entre *C. reinhardtii* y *Polytomella* sp. Las diferencias entre

las mitocondrias que fueron descritas en este trabajo podrían explicarse como una consecuencia de la pérdida de un cloroplasto funcional en *Polytomella*.

En ese trabajo, los datos bioquímicos originales confirman la relación evolutiva estrecha entre el alga incolora y el alga fotosintética, especialmente a nivel de los complejos IV y V, y de la proteína ADHE. La presencia de esta enzima anaeróbica en las dos algas no era predecible y muestra aún más claramente la cercanía filogenética de las dos algas.

Aparte de muchas similitudes, también existen sorprendentes diferencias en la composición de los complejos proteicos y en la función mitocondrial de estas dos algas. Una diferencia importante es el hecho de que el alga incolora no cuenta con una oxidasa alterna de tipo vegetal como el alga *C. reinhardtii*. La ausencia de una oxidasa alterna en el alga incolora fue demostrada inmunoquímicamente por análisis tipo Western y por mediciones de consumo de oxígeno en presencia de diferentes sustratos respiratorios y de diversos inhibidores (Reyes-Prieto y cols., 2002). También se ha mostrado que las propiedades espectroscópicas del citocromo *b* del complejo III son distintas entre las dos algas (Gutiérrez-Cirlos y cols. 1994; 1998).

La regulación de la biogénesis mitocondrial en *C. reinhardtii* y *Polytomella* sp.

Las algas clorofíceas son muy numerosas y variadas. Sólo en el género *Chlamydomonas* (incluyendo *Chloromonas*), se encuentran más de 600 especies (Pröschold y cols., 2001), la mayoría fotosintéticas pero también algunas incoloras. Los miembros de la clase Chlorophyceae se encuentran en muchos hábitats distintos, hasta en la nariz de los peces (Melkonian, 1990). En el curso de la adaptación selectiva/evolutiva, las algas han generado metabolismos distintos que les permite sobrevivir en una amplia variedad de condiciones ambientales. Se supone que las algas incoloras clorofíceas, como *Polytoma* y *Polytomella*, han perdido el aparato fotosintético (Round, 1980), posiblemente por el estrés selectivo del ambiente o bien por interaccionar con otros organismos.

El alga incolora *Polytomella* sp., que divergió de un ancestro fotosintético relacionado con *Chlamydomonas*, puede crecer en un margen muy amplio de pH ácido, y puede utilizar diferentes fuentes de carbono, como etanol, acetato, piruvato y succinato (Wise, 1955). En contraste, *C. reinhardtii* no crece a pH ácido (abajo de 5.5-6 0; eso se debe probablemente al pH óptimo del metabolismo fotosintético, vease más adelante) y puede utilizar solamente algunas fuentes de carbono. Se ha demostrado que el pH del medio de cultivo y la fuente de carbono en

Polytomella sp. tienen efectos a nivel mitocondrial (véase el inciso anterior). La expresión del citocromo *c* aumenta cuando el pH externo disminuye (Artículo II), y los niveles relativos de los complejos de la OXPHOS son diferentes (Artículo V). Estos datos representan uno de los pocos ejemplos de la regulación de la biogénesis mitocondrial en las algas. El mecanismo de esta regulación no se conoce en este momento. Datos experimentales indican que la fuente de carbono tiene una relación con el pH externo: el crecimiento en acetato de las dos algas causa una alcalinización del medio de cultivo. Esto se debe al hecho que el acético que entra a la célula es la forma protonado, dejando fuera al acetato que se forma al ajustar el pH del medio (Thomas y cols., 2002). Además, la entrada de acético acidifica el interior de la célula (Thomas y cols., 2002: Kurkdjian y Guern, 1989). El crecimiento de *Polytomella* sp. en etanol sin amortiguador causa una acidificación del medio de cultivo (Artículo II; observaciones personales). *Polytomella* sp. es capaz de ajustar su metabolismo, incluyendo el metabolismo mitocondrial, dependiendo de la fuente de carbono, y los protones claramente tienen un papel en esta adaptación. Posiblemente, *C. reinhardtii* no crece en etanol por su inhabilidad de mantener el pH adecuado o de mantener el metabolismo adecuado, a pesar de que el alga verde sí cuenta con una alcohol deshidrogenasa en el citoplasma (proteína hipotética semejante a una alcohol deshidrogenasa de zinc, contig 20021010.8185, ChlamyEST). Una diferencia importante entre las dos algas es que las mitocondrias aisladas de *Polytomella* sp. exhiben un consumo de oxígeno importante en presencia de etanol y NAD^+, mientras que las mitocondrias de *C. reinhardtii* no respiran en presencia de etanol (observaciones personales). La capacidad de *Polytomella* sp. de crecer en etanol como sola fuente de carbono podría estar relacionada con la presencia de la ADHE. No es imposible que la ADHE mitocondrial de *Polytomella* sp. funcione como alcohol oxidasa, permitiendo el consumo de etanol por la mitocondria. Si la proteína homóloga en *C. reinhardtii* se encuentra también en la mitocondria, es poco probable que la ADHE del alga verde funcione como etanol oxidasa, ya que posiblemente sea sensible al oxígeno. Se requerirán más estudios sobre las ADHE de las dos algas para establecer si efectivamente la proteína está involucrada en la respiración mitocondrial con etanol en el alga incolora.

La regulación mitocondrial por luz en *C. reinhardtii*

Uno de los objetivos de este trabajo consistió en entender mejor el efecto de la luz sobre la función de la mitocondria en las células fotosintéticas. El metabolismo de los organismos fotosintéticos, y en particular el cloroplasto como el primer sitio de la fotosíntesis, ha sido bien estudiado. Se ha reportado muchos ejemplos acerca de la regulación por luz, por medio del efecto que tiene sobre el estado redox, el metabolismo del cloroplasto y la expresión de los genes que codifican proteínas del cloroplasto (Danon y Mayfield, 1994; Allen y cols., 1995b;

Pfannschmidt y cols., 1999a,b). No obstante, en los años pasados se ha estudiado más el papel de las mitocondrias en la célula fotosintética. La mitocondria es vital, no sólo en la obscuridad, sino también en la luz, por ejemplo, en la optimización de la fotosíntesis y en el balance del estado redox celular (Artículo VI). En las plantas se sabe relativamente poco acerca de la regulación por luz de la respiración y de la biogénesis mitocondrial. Los datos disponibles muestran generalmente que el camino respiratorio de la AOX, que no lleva a la producción de ATP, aumenta en la luz, mientras que en la obscuridad es más importante el camino clásico de los citocromos (Finnegan y cols., 1997; Svensson y Rasmusson, 2001; Artículo VI). Además, se había mostrado que un componente mitocondrial de la fotorrespiración, el complejo de la glicina descarboxilasa (GDC), se activa en la luz (Walker y Oliver, 1986; Srinivasan y cols., 1995).

Las mitocondrias muestran un comportamiento diferente en distintas condiciones de luz: en la obscuridad las mitocondrias están más dedicadas a la producción de ATP, mientras que en la luz aumentan la actividad de los caminos no fosforilantes (AOX, fotorrespiración). Ya que involucran reductores, estas diferencias funcionales podrían contener la clave para los mecanismos de la transducción de la señal de la luz. Se propuso que el estado redox es importante en la regulación mitocondrial por luz, ya que este parámetro, tanto en el cloroplasto como en toda la célula, está afectado por los reductores que se forman durante el proceso fotosintético (Artículo VI).

Hasta la fecha, existen pocos datos concluyentes sobre la regulación por luz de la función mitocondrial y de la expresión de genes que codifican para proteínas mitocondriales en *C. reinhardtii*. Se ha mostrado que luz y/o el acetato aumentan los niveles del RNA mensajero del citocromo *c* (Felitti y cols., 2000) y que la expresión de los genes *ca*1 y *ca*2, que codifican para la anhidrasa carbónica mitocondrial, se inducen al aumentar la intensidad de luz (Villand y cols., 1997). También, en un régimen alternado de luz/obscuridad, los niveles del RNA mensajero del transportador de ADP/ATP mitocondrial (CRANT) se encuentran altos en la fase de obscuridad y bajos en la fase inicial de luz (Sharpe y Day, 1993). Recientemente, se ha publicado un estudio de microarreglos de ADN donde se monitoreó el efecto de varias condiciones de cultivo sobre la expresión de genes en *C. reinhardtii*, incluyendo el crecimiento en la obscuridad (Lilly y cols., 2002). En ese trabajo no detectaron mayores diferencias entre el crecimiento en la luz y en la obscuridad para la expresión de los genes que codifican para componentes mitocondriales como la subunidad COXIII, la oxidasa alterna, el citocromo *b* y la proteína H del GDC.

C. reinhardtii se ha usado extensamente como modelo para la fotosíntesis, pero desde ese punto de vista es un organismo un poco excéntrico, porque se mantiene verde en la

obscuridad. Sería interesante ver si éso tiene implicaciones en la regulación mitocondrial por luz en *C. reinhardtii* y si las respuestas mitocondriales a la luz son comparables con las respuestas que se observan en las plantas. En este trabajo se obtuvieron algunos resultados al respecto; se incluyen como figuras en la sección de los resultados adicionales. Los datos adicionales de los análisis de hibridaciones tipo Northern y Western no fueron consistentes, y por lo tanto no fueron incluidos. Aparte de la recomendación de usar la cepa silvestre en lugar de la CW15, también es de vital importancia contar con condiciones de luz y temperatura constantes.

Efecto de la luz y el acetato sobre el crecimiento y la ultraestructura de C. reinhardtii

La luz tiene un efecto muy importante en el crecimiento de *C. reinhardtii*. En la luz, el alga puede alcanzar altas densidades (hasta $2.5x10^7$ células/ml), independendientemente de la cantidad de acetato en el medio de cultivo. En la obscuridad, el crecimiento del alga depende de la concentración del acetato (Figura I, resultados adicionales); a concentraciones menores de 50 mM de acetato, la fuente de carbono se vuelve limitante en la obscuridad. Con una concentración de 50 mM de acetato (medio H3), el crecimiento en la obscuridad fue comparable con el crecimiento en la luz. A diferencia de las plantas, las células de *C. reinhardtii* crecidas en la obscuridad retienen una parte de la clorofila. Esto puede servir para mantener el cloroplasto listo para reiniciar la fotosíntesis rápidamente cuando regresa la luz. Se ha observado que en *C. reinhardtii*, la presencia de acetato en el medio de cultivo disminuye la participación de la fotosíntesis en el metabolismo celular en condiciones de iluminación (Heifetz y cols., 2000). De acuerdo con esos datos hemos observado que la intensidad del color verde de las células crecidas en el medio H3 (50 mM de acetato) en la luz es considerablemente menor que lo que se observa en el medio TAP (17 mM de acetato). Sin embargo, la luz sí permite al alga crecer hasta densidades altas, aún en concentraciones altas de acetato (Figura I, resultados adicionales). Además, en la obscuridad las células se mueren mucho más rápidamente después de llegar a la densidad máxima del cultivo.

Las imágenes de microscopía electrónica de células de la cepa CW15 (que carece de pared celular), crecidas en la luz y en la obscuridad en el medio TAP y H3 muestran por lo menos dos diferencias claras. Primero, en la luz, la alta concentración de acetato y la obscuridad disminuyen drásticamente la acumulación de almidón en el cloroplasto igual que en las plantas, mientras que el cloroplasto mismo parece estar menos desarrollado. Segundo, la cantidad de mitocondrias parece ser más alta en células crecidas a altas concentraciones de acetato, especialmente en la obscuridad (Figura J, resultados adicionales). Entonces, el acetato tiene un efecto sobre el tamaño y el desarolio del cloroplasto y sobre la acumulación de almidón, y

además parece disminuir la capacidad fotosintética. También, la obscuridad aumenta la capacidad respiratoria, considerando que la cantidad y en algunos casos, el tamaño de las mitocondrias es más grande en la obscuridad, independiente de la concentración de acetato en el medio. Todas estas observaciones fueron reproducibles, pero la cuantificación de las diferencias en la acumulación de almidón, el estado de desarollo del cloroplasto, y la cantidad de mitocondrias en la célula, exhibieron cierta variabilidad experimental. Sin embargo, la tendencia observada fué como la que se mencionó anteriormente.

Los datos obtenidos en la luz y obscuridad con acetato pueden tener que ver con el hecho de que se acidifica el medio interno de la célula, ya que a la entrada del ácido acético en el citoplasma se disocia por el pH ligeramente básico en el mismo. Esta acidificación podría tener efectos adversos sobre la fotosíntesis. El pH óptimo en el estroma de los cloroplastos para la fotosíntesis es de 7.8, y una acidificación del estroma causa la inhibición de la fotosíntesis (Heber y cols., 1994). Además, la obscuridad puede causar ciertos niveles de anaerobiosis en las células por la falta de generación del oxígeno liberado por el fenómeno fotosintético (Klock y Kreuzberg, 1991). Condiciones anaeróbicas causan la acidificación del citoplasma (Kurkdjian y Guern, 1989), y podrían tener efectos aún más profundos en el metabolismo de *C. reinhardtii*.

La luz y el perfil de proteínas mitocondriales en BN-PAGE

Para aislar las mitocondrias de las células crecidas en la luz y en la obscuridad, se utilizó principalmente el medio H3, ya que la cantidad de células que se obtienen con el medio TAP en la obscuridad es muy baja. Las mitocondrias puras obtenidas de células de la cepa que carece de pared celular (CW15), crecidas en medio H3 en la luz y en la oscuridad, fueron solubilizadas con *n*-dodecil maltósido al 1% y aplicadas a geles BN-PAGE. La segunda dimensión desnaturalizante reveló las subunidades de los complejos, además de otras proteínas (Figura K, resultados adicionales). Ya se sabe que la luz influye sobre la cantidad de mitocondrias; en BN-PAGE, se analizaron mitocondrias puras, y no la cantidad de estos organelos en las células. Se utilizaron cantidades iguales de proteínas mitocondriales para el análisis en BN-PAGE (hasta 1000 µg), por lo que las diferencias observadas en la Figura K reflejan diferencias en la composición de las mitocondrias, no en su cantidad.

Una proteína que presenta una acumulación diferencial en la luz y en la obscuridad es el transportador de ADP/ATP (CRANT). En la luz, la cantidad de esta proteína hidrofóbica es mayor que en la obscuridad; la explicación más sencilla es que aparentamente, en la luz más ATP está siendo exportado de las mitocondrias. Es posible que por la alta concentración de acetato (50

mM), una parte importante de la demanda de ATP en el cloroplasto, por ejemplo para la síntesis de aminoácidos, venga de las mitocondrias, ya que se sabe que la fotosíntesis está parcialmente inhibida en estas condiciones. Para saber el papel del acetato en está regulación, se tendría que analizar por BN-PAGE a las mitocondrias de células crecidas en el medio TAP, en distintas condiciones de luz.

El análisis en BN-PAGE reveló otro ejemplo interesante de acumulación diferencial. Se trata de un complejo proteico que migra en geles azules arriba del complejo V, con una masa molecular grande difícil de estimar. En la segunda dimensión, este complejo de varios miles de kDa se resuelve en dos bandas de aproximadamente 80 y 45 kDa. La movilidad electroforética de la banda en los geles azules nativos indica que el complejo es multimérico. Por su tamaño y su composición polipeptídica, esta proteína probablemente coresponda al complejo de la piruvato deshidrogenasa (PDC), con dos de sus tres subunidades mayores, E2 y E1. En la papa, las subunidades principales E1, E2 y E3 tienen un peso molecular de 43, 78 y 58 kDa (Miliar y cols., 1998). Anteriormente se había analizado el PDC en geles azules nativos, usando agarosa en lugar de poliacrilamida, para permitir la migración de este complejo de gran tamaño. Para evitar la disociación del PDC, se tuvo que usar bajas cantidades de sales en las muestras y los geles azules (0.05 M en lugar de 0.5 M de ácido aminocaproico), ya que las subunidades del complejo PDC no están unidas covalentemente (Henderson y cols., 2000). Es probable que por la concentración de sales usadas al correr los geles azules nativos en este trabajo (Apéndice II), una parte del complejo PDC se haya disociado, dejando únicamente a las subunidades E1 y E2 unidas. Henderson y colegas (2000) reportaron que el complejo PDC no entró a geles de poliacrilamida de 3 o 4%; el complejo PDC completo parece ser demasiado grande como para entrar en el gel y por eso no es visible. Sin embargo, un subcomplejo de E1 y E2 de varios miles de kDa sí parece entrar al gel (4% de acrilamida).

En las mitocondrias aisladas de células de *C. reinhardtii* crecidas en el medio H3, la PDC es más abundante (Figura K, resultados adicionales) y sugiere un mayor nivel de respiración mitocondrial en la obscuridad que en la luz. En el medio TAP, la PDC no se detecta en la luz en BN-PAGE pero sí se encuentra en mayor abundancia en la obscuridad (no mostrado). La expresión de la PDC también está aumentada en la obscuridad en mitocondrias de plantas (Budde y Randal, 1990). La pregunta es qué papel tiene la PDC en el metabolismo de acetato; en *C. reinhardtii*, el acetato entra el ciclo de glioxilato y forma luego fosfato de triosa (Heifetz y cols., 2000): el acetato se convierte en acetil-CoA en el glioxisoma por la acetil-CoA sintasa (ACS) (Eastmond y Graham, 2001). Luego, la acetil-CoA se une con el glioxilato para formar malato y después succinato, en el ciclo de glioxilato. El succinato se importa al interior de la mitocondria y se convierte en malato en el ciclo Krebs. El malato puede salir de la mitocondria

para finalmente formar fosfatos de triosa y sacarosa. El malato también puede ser convertido en piruvato por la enzima málica de la mitocondria y por la piruvato cinasa a partir de fosfoenol piruvato (Eastmond y Graham, 2001). Como la ACS, el PDC también produce acetil-CoA por la conversión de piruvato en acetil-CoA, por lo que deberá considerarse también el metabolismo de piruvato en relación con el del acetato.

Los perfiles de las proteínas mitocondriales en la luz y en la obscuridad no presentan grandes diferencias en la acumulación de los complejos de la OXPHOS. Es posible que la regulación por luz consista en un recambio más veloz de las proteínas, o esté presente a nivel de la activación/desactivación de los complejos. Existen indicaciones de que en las plantas sucede esta segunda posibilidad (Artículo VI). La AOX probablemente esté regulada por la luz a nivel de la acumululación de la proteína, pero en nuestro caso, no se obtuvieron resultados consistentes acerca de la regulación de la AOX. Los resultados preliminares parecen indicar que la AOX se expresa más en la obscuridad y a altas concentraciones de acetato. En las plantas, la AOX se expresa más en la luz, especialmente en condiciones fotorrespiratorias (a bajos niveles de dióxido de carbono). Seguramente la regulación mitocondrial por luz será investigada más profundamente en el futuro.

Varias proteínas mitocondriales más se expresan en forma diferente en la luz y en la obscuridad en *C. reinhardtii*, pero no han sido todavía identificadas. Lo que es cierto, es que la luz tiene efectos importantes sobre las mitocondrias del alga verde, lo que confirma la participación activa de estos organelos en la fotosíntesis. En general, las condiciones experimentales modifican la expresión de las proteínas y que por lo tanto se convierten en una herramienta poderosa para entender como las células de *C. reinhardtii* y *Polytomella* sp. controlan su metabolismo.

Perspectivas

En el caso de *C. reinhardtii*, la disponibilidad de la secuencia de nucleótidos del genoma nuclear, así como la base de datos de los cDNAs (ChlamyEST), permitirá eventualmente el análisis y la validación de las secuencias de todos los genes. El primer análisis de microarreglos, usando clonas de cDNA conocidas, fue publicado recientemente (Lilly y cols., 2002). Sin duda, esta metodología facilitará la disección del metabolismo de *C. reinhardtii* y la organización y función del sistema genético y bioquímico. Sin embargo, el análisis bioquímico es indispensable para evaluar la estructura, función e interacción de los productos de los genes. En esta tesis se han reportado principalmente datos bioquímicos que proporcionan una base descriptiva para

continuar con los estudios de la regulación mitocondrial así como de la biogénesis y de la estructura mitocondrial en las algas. La presencia de proteínas 'misteriosas' como la ADHE y la MASAP demuestra que las mitocondrias de las algas no sólo exhiben características extraordinarias, sino también hace posible que se identifiquen otras funciones no contempladas previamente. Se sugiere que las mitocondrias pueden evolucionar para ampliar sus funciones, por ejemplo por la transferencia lateral de genes de otros organismos. Un mejor conocimiento de la composición, función y regulación mitocondrial podría explicar las razones bioquímicas y fisiológicas por las cuales las algas pierden, adquieren, o retienen ciertas funciones en sus mitocondrias. Seguramente, el alga verde y su 'prima cercana' heterotrófica nos dan una buena oportunidad de estudiar en un mismo marco metabólico los beneficios de la fotosíntesis y las adapciones en la ausencia de la misma.

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APÉNDICES

APÉNDICE I

SECUENCIAS

Las secuencias obtenidas en este trabajo se depositaron en la base de datos DDBJ/GenBank™/EBI Data Bank con los siguientes números de acceso:

AF411119	- secuencia de traducción (cDNA) del gen *atp6* de *C. reinhardtii*
AF411921	- secuencia genómica del gen *atp6* de *C. reinhardtii*
AJ441255	- secuencia de traducción (cDNA) del gen *masap* de *C. reinhardtii*
AF245393	- secuencia de traducción (cDNA) del gen *cyc*$_1$ de *C. reinhardtii*
AJ417788	- secuencia genómica del gen *cyc1* de *C. reinhardtii*
AJ320239	- secuencia genómica del gen *isp* de *C. reinhardtii*
AJ495765	- secuencia de traducción (cDNA) del gen *bt-aad* de *Polytomella* sp.

Otras secuencias obtenidas a partir de la base de datos ChlamyEST:

- Los contigs son secuencias ensambladas a partir de las secuencias de ChlamyEST, obtenidas en el sitio de la red con la dirección (www.biology.duke.edu/chlamy_genome/cgp.html).

- El aminoácido que marca el inicio de la secuencia amino terminal esté en grueso y subrayado, el asterisco indica el codón de término.

- Los pesos moleculares mencionados ariba de cada secuencia se refieren los mencionados en el artículo I

- Para obtener secuencias a partir de los numeros de contig, se puede ir al sitio

http://www.biology.duke.edu/chlamy_genome/search.html,

seleccione la categoría 'ACEs', entre el número de contig y oprima 'Search'.

Complejo I

Subunidad de 51 kDa, contig 20021010.2731.1

51 kDa
MQRTGGLVSQLAGAQLTGALQELKTGVLRAFSTAAPAAGAPPPPPPPPAKTSFGGLKDEDRIFQNIYGRHDLSIKGAMS
RGDWYMTKEIIGKGRDWIIDQMKKSGLRGRGGAGFPSGLKWSFMPKASDSRPIYLVVNGDESEPGTCKDREIMRHEPHK
LVEGCLMAGVAMGARAGYIYIRGEFVQERRAVERAISEAYAKGFLGKNACGSGVDFDLMVHYGAGAYICGEETALIESL
EGKQGKPRLKPPFPAGVGLYGCPTTVTNVETVAVSPTILRRGPEWFSSFGRKNNAGTKLFCISGHVNRPVTVEEEMSIP
LKELIERHAGGVRGGWDNLLAIIPGGSSVPLLPKKICDGVLMDFDALKEAQSGLGTAAVIVMDKSTDVIDAIARLSYFY
KHESCGQCTPCREGTGWLYDIMTRMKKGDARLEEIDMLWEITKQIEGHTICALGDAAAWPVQGLIRHFRGEMEERIKSA
GGKKKLAATA*

Subunidad desconocida, contig 20021010.2648.1

29 kDa
MLKRVGQSLVPFARAGLTQTAESFRGVSSQFFDAPNGPSVKQVLIEDEWYNRQRSIFPLLDKEPYYPVDVFVAPNAVVC
GDVDIYGGASVFFGAVLRGDLNKIRLGNRSAILDRAVVHAARAVPTGLNAATLIGEKVTVEPYAVLRSCRVEPKVIIGA
RSVVCEGAVVESESILAPNSVVPPARRIPSGELWGGSPAKFIRKLTDHERDRVLDDVSTHYHNLATMFRREALEPGTGW
RDVEAWRQKLVDQGEFQWINSREQKYLMPPAARGPRRLEKLTH*

Subunidad de 24 kDa, contig 20021010.5518.3

28 kDa
MLSRALLLAGRLAATGQQQAASTSSRAVQPLGSLLQRCNFATNSTDIFNIHKDTPENNAATSFEFSEATLKVVNDIIAR
YPPNYKQSAIIPVLDVTQQENGGWLSLAAMNRVAKLLDMAPIRVYEVATFYTMFNRTKIGKYHVQICGTTPCRLQGSQK
IEEAITKHLGIGIGQTTQDGLFTLGEMECMGACVNAPMVAIADYTKGVSGFEYIYYEDLTPKDIVNILDTIKKGGKPKP
GSQYRLKAEPAGAVHGGEKWVPKDGETTLTGAPRAPYCRDLNATA*

Complejo III

Proteína estructural I (subunidad beta de la peptidasa procesadora mitocondrial), contig 20021010.2906.1

53 kDa
MRSLKQILRIGEASSLGLRAFGSAAKDVVATDANPFLRFSNPRPSPIDHTPLLSTLPETRITTLPNGLRVATEAIPFAE
TTTLGIWINSGSRFETDANNGVAHFLEHILFKGTKNRSVKELEVEVENMGGQLNAYTGREQTCYYAKVMGKDVGKAVNI
LSDILLNSNLDARAIDKERDVILREMEEVNKQTSELVFDHLHATAFQYSPLGRTILGPVENIKSINRDQLVEYMKTHYR
GPRMVLAAAGAVNHDELVKLASDAFGSVPDEDAATSVRSLLVKEPSRFTGSYVHDRFPDASECCMAVAFKGASWTDPDS
IPLMVMQTMLGGWDKNSTVGKHSSSALVQTVATEGLADAFMAFNTNYHDTGLFGVYGVTDRDRSEDFAYAIMSNLTRMC
FEVRDADVARAKNQLKASLMFFQDSTHHVAESIGRELLVYGRRIPKAEMFARIDAVDANAIRAVADRFIYDQDMAVASA
GDVQFVPDYNWFRRRSYWLRY*

Proteína estructural II (subunidad alfa de la peptidasa procesadora mitocondrial), contig 20021010.3079.1

48 kDa
MLGSSTSQLAPAMVRSIASSAAASTAAPVLAAKSGGLLASVFGMGGGRVEVPLSEKLPAVTEPPRTSTPATKPIVQTSS
LRSGVKVASINTVSPISSLVLFVEGGAAAETPATAGASKVLEVAAFKATANRSTFRLTRELEKIGATSFARAGRDHVAF
GVDATRLNQLEALEILADAVVNARYTYWEVRDSLDAVKEQLAAQLRNPLTAVNEVLHRTAFEGGLGHSLVVDPSVVDGF
TNETLKEYVHSIMAPSRVVLAASGVDHAELTALATPLLNLHGNAHPAPQSRYVGGAMNIIAPTSSLTYVGLAFEAKGGA
GDIKSSAAASVVKALLDEARPTMPYQRKEHEVFTSVNPFAFAYKGTGLVGVVASGAPGKAGKVVDALTAKVQSLAKGVT
DVQLATAKNMALGELRASVATAPGLAAGGGLQRAGDGQVQRERGGGGAVGPDGGGRDQLRERHD*

Complejo IV

Subunidad COXVIb, contig 20021010.4777.1

16 kDa
MGLFNYFVARADAEVVEEEHAPPPPPPPPKKSSRKPTLESLSADELEELKNEVVSEVVDKIAGEDGTKLADFLEPELIT
APYDPRFPNRNQARHCFVRFNEYYKCLYERGEEHPRCQFYQKAYQSLCPSEWVESWQELREKGLWTGKY*

Subunidad COXVb, contig 20021010.5550.2

13 kDa
MNRLGALSGLLARAARTCSRRWATAASGVPAELSAVGIVGQEFAAQARSLHTSLTTCQGAPAEAKPSALSAEPPRKYRP
LGDKELWHEAWMYEDKFGTEEDPIIVPSLEAERIIGVTDPEDETLVVWGILKDGEPPRQFVENGEFYVLKHVEYIKKVG
DVLEAIEGGADKAKIAK*

Subunidad COXVIa, contig 20021010.1171.2

12 kDa
MQALRRAVSTAMPGFRRASTTAGETIDKYWAPYFPKPAVTADEAKKSVNKEMVGFMLLGPVGVAFMLYDFAVGLEEEHH
VTIPPYPWMRIRRLPGMPWGQDGLFEGHPRVATTWPPEEGAADSHH*

Subunidad COXVIIc, contig 20021010.7901.1

9 kDa
MSSALRRLSQQAPRLTRGLKTGNVTKGGAEKYSHEEVVYGDGHHGLRKGYTYDFEHGPHYLQPEKIPNFWSKFYAGTGA
LYAVGLGVPLFAVWWQQSKLKA*

Complejo V

Subunidad del complejo V desconocida, contig 20021010.1861.2

45 kDa
MRSAAVRVLGAQWAGVGAQEAGSRAARAFATATFVPGVSGDASGVVSAVDALMSHDSAATGKDVADAAVALAYLGTRGN
RRVWGKVLEKAASTPLDGPSLANLSWALSAANVDHTRTLAELAGPLAASLKSLSPAQVSFAVEAVGKSGAADVELFAA
VTELAAARTADFKAADLARLLWGFGAAGVQDGKLVKAASAGLVAKAAELGGREAAQALWGLAALRRVPDAALAGALTKA
LKAGVEAPADAAAAAWALATLAVKADAGTVKALADKAKAGVADLSAAQAVQGGWGLAMLGDKDGAAALLGAAAAAVQKD
PTSLSPSALALLHAGAVVSGAGLPNPVSDFAAKGFGLAVEHGRHSRRSAAAAFHAKLAEAVAYANGARHRPDVASKVAS
FVSSGPDGSTLDVVVPADANTKLAVLGVEAEALASNGAVLGGSLAAARVREAQGFKVAVVPQTEFPTGAPLKQRAAAVL
GAIKKAVPGLSAMADKLSREL*

Subunidad del complejo V desconocida, contig 20021010.1014.1

38 kDa
MLRKGAQAVLQAERAGPQQTCAAAQTAFTRQFGAPAGSHDHPTTPLSPIMPGIVAIPRQVISTAASLTGKAVAGAATSS
TIRDLVTSFAEKAIISESIVKVDEVDVPFWAYWLSTAGYNSPAGFKKFAEAVKPKVAGLEPQQVTDLVVAFHKVNYFDK
DLFAAVAANISANFTKYETEQLLQVLSAFVEFGFYDATAYDDIADSITYCNHYLAPVRACPSQLASAFAAFAKYEHERG
DLFVALARGFSELSLAKLGAEERKGTVLKALRAFHRFNFWPDATEALLHAAKGLEGSLSADEAKEVEKYQKLLEDAAGG
EFKVFKEGDDVDGVHWYGHHTQAPTGYSLYVFREALVPKQYSPASMRPIK*

Subunidad del complejoV desconocida, contig 20021010.8373.1

35 kDa
MASGLLRSLGVLSRNCAGSVQEGAVRAFATGAAPSKKDVLYNLSNPDPDAEASVKAYLTSLYKGAKLEPTTADDSLELT
SKIEKKYKAAAIVEYGLQTISVPLGYSKSDLAPVKPYAAELPSLAKQAGFEDPATEVSKRLGATAATADSVKELLSKNQ
SLMSADLYAALSEAVQQVENATNATLTLDGASPAYKQFAAKVEAIAKAHGIPAKLLVDVKKGAADEATSDALAKEYARW
QQHAAVKDAIAELEALKAEATAVLDKHLGKTAEQVRSEQAAVLAAAIKKAEAAKGAPWAAAFLEDVKKVQWFDACVAEN
PAVGPKVTA*

Subunidad gama, contig 20021010.5435.1

31 kDa
MALRNAASFLGKSLAGAAELGFSAAKTAGGEALTNAFVTDGVRHASNQAVKQRIRAIKNIGKITKAMKMVAASKMKNAQ
VAVEQSRGIVNPFVRLFGDFPAIEGKQNITVAVSSDRGLCGGLNSNIAKYTRALLKMDPTTSETTKLVSIGDKGRSQLM
RTNGEMFTHTFSETYKVRVTFAQASLIAEDLLKSNPEAVKILFNKFRSAISFKPTLATILTPETLEKQLTEPSGNRLDA
YEIEASHERSDVLRDLAEFQLAATLYNAMLENNCSEHASRMSAMENSTKSAGEMLGKLTLEYNRKRQATITTELIEIIA
GASALMDA*

Subunidad delta o subunidad que confiere sensibilidad a la oligomicina (OSCP) (Oligomycin sensitivity conferral protein), contig 20021010.9342.1

24 kDa
MLARAACLLARSAEQAQLPQIMVRTFAAAAAAKTAPKAEMKLPVAPLQLSGTSGAIATLAWQVAAKENVLAKVQDELYQ
LVEVFKSHPEIRRLATDPFLPDAFRRKVVRDMFATKDVTEVTKRLVEALAEENSLSAIVQVTLAYEELMLAHKKEVHCT
VVTAQPLDDAERAVFTKQAQAFVDPGFKLVMKEKVDRKLLGGFVLEFEDRLVDMSQAKKLEEFNNLVPKLENDLK*

Subunidad del complejo V desconocida, contig 20021010.2291.1

19 kDa
MQRSTELVGAFQRAGAALASPAASRQLSTLVEKFTFGSAADGPTASLGSNVKLTVKGSGKGVDVSVSAGAGSAKVSYAP
SDLRKVAASSLVLQDVSRISTAHSAFMNYLLTLTHERYSVLATWPDFTKAYGKDYYYRAHPDDLRKFYSMVDEFHRMWD
VVTEFGSLSGLASQLVPGYRVRRHNTVHPALGPATADGAVVQFLLAHAK*

FALTA

PAGINA

100

APÉNDICE II

MATERIALES Y MÉTODOS

1. 2D ELECTROFORESIS EN GELES NATIVOS AZULES (BN-PAGE)

Los geles nativos azules se pueden correr en casi cualquier cámara de electroforesis para proteínas. Las condiciones para preparar y correr la muestra dependen del tipo de organismo, y si se utilizan proteínas solubles u organelos enteros. Para analizar las mitocondrias de las algas unicelulares, se tiene que cargar una cantidad grande de material (alrededor de 1 mg de proteínas), lo que puede a veces resultar en la precipitación de las proteínas en el pozo del gel. Para mejorar la resolución de las mitocondrias del alga fotosintética, las muestras se aplicaron frescas o guardadas en hielo durante unos cuantos días. No se recomienda congelar las mitocondrias intactas ni las mitocondrias ya solubilizadas, pues la resolución electroforética ya no es óptima. Los geles se pueden preparar un día antes de correr las muestras, protegidos en papel plástico (Ega-Pack) y guardados a 4°C.

1.1. Gradiente para geles azules nativos

Para cámaras de electroforesis GibcoBRL Vertical (VI6) (1.5 mm de espesor)

	Acrilamida (H%)			Acrilamida (L%)		gel concentrador
	10%	12%	15%	5%	8%	4%
48.5 % acrilamida*						
1.5 % bis-acrilamida	3.8 ml	4.6 ml	5.7 ml	1.9 ml	3.07 ml	0.50 ml
amortiguador para el gel 3x		6.2 ml			6.2 ml	2.0 ml
glicerol 80%		4.73 ml			1.2 ml	-
agua destilada	3.8 ml	3.04 ml	1.9 ml	9.2 ml	8.1 ml	3.44 ml
TEMED		18 µl			18 µl	9 µl
persulfato de amonio (10 %)		50 µl			50 µl	32 µl
En el formador de gradientes		16.5 ml			16.5 ml	

Para cámaras de electroforesis BioRad Mini Protean II o Hoefer Mighty Small (1.5 mm de espesor)

	Acrilamida (H%)			Acrilamida (L%)		gel concentrador
	10%	12%	15%	5%	8%	4%
48.5 % acrilamida*						
1.5 % bis-acrilamida	0.72 ml	0.87 ml	1.08 ml	0.36 ml	0.58 ml	0.25 ml
amortiguador 3x		1.17 ml			1.17 ml	1.0 ml
glicerol 80%		1.0 ml			0.25 ml	-
agua distilada	0.6 ml	0.44 ml	0.23 ml	1.7 ml	1.5 ml	1.75 ml
TEMED		6 µl			6 µl	6 µl
APS (10 %)		18 µl			18 µl	18 µl
En el formador de gradientes		3.5 ml			3.5 ml	

* La solución de acrilamida 48.5 % puede cristalizar a 4°C.

El gradiente de acrilamida se debe ajustar al tamaño de las proteínas o complejos proteicos que se desean estudiar. En el caso de los complejos proteicos de más de 100-kDa, se recomienda usar un gradiente de poliacrilamida del 5 al 12 %, mientras que un gradiente del 6 al 18 % es más adecuado para proteínas en el rango de 20 a 500 kDa. Alternativamente, también pueden utilizarse geles de una sola concentración (12 o 15%).

Preferentemente, usar un formador de gradientes con dos cilindros separados. El gel concentrador pude ser pequeño: 0.5 cm abajo de los pozos en el caso de los geles chicos y 1.0 cm para los geles grandes. Es preferible colocar el gel concentrador imediatamente sobre el gel de separación sin esperar que este último polimerice; esto asegura una corrida constante.

PREPARACIÓN DEL GEL

- Preparar las soluciones para el gel de separación y para el gel concentrador en presencia del TEMED. El APS se agrega después.
- Poner el formador de gradientes sobre un agitador magnético arriba de una plataforma ajustable (Jiffy Jack), la salida del formador de gradientes debe estar al menos 20 cm por arriba de las placas de vidrio.
- Cerrar las dos válvulas del formador de gradientes antes de agregar las soluciones. Para facilitar el flujo de las soluciones, conectar un jeringa al tubo de salida.
- Agregar la solución de alta concentración de acrilamida (H%) en el cilindro más cercano a la salida y poner una mosca magnética.
- Agregar el APS a la solución de baja concentración en acrilamida (L%), mezclar, y agregar en el otro cilindro del formador de gradientes. De aquí en adelante debe trabajarse rápidamente, para preparar el gradiente del gel antes de que cualquiera de las soluciones polimerice en el formador de gradientes o en la tubería.
- Agregar el APS a la solución de alta concentración de acrilamida, y mezclar fuertemente con la mosca
- Bajar la velocidad de la mosca (mezclar fuertemente impide el flujo de la solución de baja concentración en la solución de alta concentración de acrilamida).
- Abrir la válvula entre los dos cilindros.
- Remover las burbujas de aire atrapadas entre los cilindros.
- Abrir la válvula de salida y succionar el líquido con la jeringa con mucho cuidado, hasta que el líquido llegue cerca de la punta de la jeringa.
- Retirar la jeringa del cilindro de salida y poner el tubo imediatamente entre los vidrios.
- Asegurarse que los nivel de las soluciones (en los dos cilindros) bajen a la misma velocidad. Bajar la velocidad de la mosca si necesario.
- En caso de agregar la solución del gel concentrador imediatamente: agregar el APS a la solución y colocarla con cuidado sobre el gel de separación. Poner el peine.
  Nota: si no se quiere agregar el gel concentrador de inmediato, poner un poco de agua arriba del gel de separación. Una vez que el gel ha polimerizado, quitar el agua y agregar la solución para el gel concentrador y poner el peine.

Lavar de inmediato el formador de gradientes, antes de que polimericen los residuos de las soluciones y tapen la tubería.
Remover con mucho cuidado el peine del gel sin dañar los pozos.
Quitar el agua de polimerización sacudiendo las placas. Limpiar los vidrios con papel absorbente.
Si el gel no se va a usar imediatemente, dejar el peine, envolver en papel plástico (Ega-Pack) y guardar a 4°C.

SOLUCIONES

Nota: En cada amortiguador, se puede cambiar el Bis-Tris por Imidazol (agregando solamente la mitad de la concentración).

Amortiguador de geles 3x (100 ml)				
ácido amino-caproico	1.5	M	19.68	g
Bis-Tris (MW=209.2) / Imidazol	150 / 75	mM	3.14 / 0.51	g
Ajustar el pH a 7.0 con HCl				
Amortiguador del cátodo (superior) 1 (1 L)				
Tricina	50	mM	8.96	g
Bis-Tris / Imidazol	15 / 7.5	mM	3.14 / 0.51	g
Azul de Coomassie Serva G	0.02	%	200	mg
Amortiguador del cátodo (superior) 2 (1 L)				
Tricina	50	mM	8.96	g
Bis-Tris / Imidazol	15 / 7.5	mM	3.14 / 0.51	g
Azul de Coomassie Serva G	0.002	%	20	mg
Amortiguador del anódo (inferior) (1 L)				
Bis-Tris / Imidazol	50 / 25	mM	10.46 / 1.7	g
Ajustar el pH 7.0 con HCl				

Para prevenir la agregación del azul del Coomassie Serva G que a su vez conduciría a la agregación de las proteínas, es importante evitar la presencia de iones bivalentes en los amortiguadores. La solubilidad del azul de Coomassie es crítica; no debe haber precipitados.

Preferentemente usar el Coomassie Serva Blue G; el Coomassie Blue 250R or G de otra marca (como Sigma, por ejemplo) sólo se puede usar si ha demostrado ser completamente soluble en el amoriguador del cátodo.

1.2. Preparación de la muestra

Para BN-PAGE, las mitocondrias de las algas unicelulares se solubilizan con *n*-dodecil-β-D-maltósido durante 30 min en hielo. Para eliminar el material no solubilizado, se ultracentrifugan las muestras. El azul de Coomassie se agrega después para proporcionarle una carga negativa a las proteínas. Eso aumenta la movilidad electroforética y permite una mejor separación de las proteínas en comparación con otros geles nativos.

Las proteínas solubles se preparan para BN-PAGE agregando directamente el mismo detergente a una concentración final del 1%. Considerando que una relación fija (4:1) entre la cantidad de detergente y de Azul de Coomassie, se tendrá que agragar a la muestra de proteínas solubles el Azul de Coomassie correspondiente.

1.2.1. Mitocondrias y fracciones membranales

Como se mencionó anterioremente, los mejores resultados se obtuvieron con muestras preparadas imediatamente antes de cargarlas en el gel.

Los pasos para preparar las mitocondrias para Gel Azul Nativo son los siguientes:

- Centrifugar las mitocondrias at 4 °C (durante 5 min a la máxima velocidad en una microcentrífuga de mesa). Resuspender la pastilla en amortiguador de lavado, volver a centrifugar 5 min y remover el sobrenadante.
- Resuspender las mitocondrias en el amortiguador para muestra. Por cada mg de proteínas mitocondriales, el volumen final será de ~ 200 µl. Para calcular el volumen del amortiguador de muestra que debe agregarse en la resuspensión, tomar en cuenta el volumen de la pastilla y los volúmenes de dodecil-maltósido y de amortiguador con Coomassie que se agregarán posteriormente. Por ejemplo: una pastilla de 1 mg de proteínas mitocondriales es de aproximadamente 25 µl, como el lauril-maltósido se agregará a una concentración final de 1% a partir de una solución al 10% (20 µl); la cantidad de colorante que se debe agregar representa ¼ de la cantidad de detergente, por lo que se agregarán 10 µl de una solución de azul de Coomassie al 5%. Finalmente, se tendrán que agregar 145 µl de amortiguador de muestra (200 - (25+20+10)). Así, el volumen total de la muestra será de 200 µl.
- Agregar el lauril-maltósido a la muestra y solubilizar invirtiendo el tubo y agitándolo en el Vórtex suavemente. A menos que haya material insoluble como almidón, la muestra debería volverse completamente transparente. Si esto no sucede, como se observa con frecuencia con pastillas de membranas mitocondriales, se tendrá que resuspender la solución con una pipeta, dejándola más tiempo en hielo y/o agregando más detergente. En general 2 g de lauril-maltósido / g proteína son suficientes para lograr una buena solubilización, pero en ocasiones se requiere más detergente. En el caso de las mitocondrias de *Polytomella* sp. se requieren hasta 4 g de detergente/g de proteína (ver la figura que se muestra más adelante).
- Ultracentrifugar a 100,000 x *g* a 4 °C, 20 min (33,000 rpm en el rotor 50Ti)
- Agregar el amortiguador con Coomassie Serva Blue G al sobrenadante y mezclar por inversión. Guardar en hielo.

  Nota: el volumen después de la ultracentrifugación puede bajar un poco debido al vacío.

1.2.2. Proteínas solubles

- Para cargar fracciones solubles con un alto contenido de proteínas se puede usar la solución "well mix HB" (vea abajo). Para cargar fracciones solubles con un contenido en proteínas bajo se usará la solución "well mix LB" (vea abajo).
- Correr con bajas cantidades de azul de Coomassie, ayudará en ver mejor las proteínas de baja masa molecular.

SOLUCIONES

Amortiguador de lavado **(100 ml)**

sorbitol (MW= 182.4)	250	mM	4.56 g
Bis-Tris / Imidazol	50 / 25	mM	1.05 / 0.17 g

Ajustar el pH a 7.0 con HCl

Aamortiguador de muestra **(50 ml)**

ácido amino-caproico	750	mM	4.92 g
Bis-Tris / Imidazol	50 / 25	mM	0.52 / 0.09 g

Ajustar el pH a 7.0 con HCl

Solución de dodecil-maltósido 10% **(1 ml)**

Dodecil-maltósido	10	%	100	mg

Disolver en 1 ml H_2O

Solución de Coomassie Serva Blue G **1 ml**

Azul de Coomassie Serva G	5	%	50	mg

Disolver en 1 ml de amortiguador de muestra

Mezcla para los pozos **1ml**

HB: amortiguador de muestra	850	µl
Dodecil-maltósido 10%	100	µl
solución de Coomassie Serva G	50	µl
LB: amortiguador de muestra	985	µl
Dodecil-maltósido 10%	10	µl
solución de Coomassie Serva G	5	µl

1.3. Cargado y corrida del gel

La electroforesis se corre en general a 4°C, por lo cual es importante asegurarse de que todos los amortiguadores estén equilibrados a esa temperatura. Armar la cámara y las placas y dejar enfriar (4°C).

Para facilitar el cargado de las muestras, agregar el amortiguador del cátodo solamente en los pozos. Si se cargan muestras con el "well mix" LB, es preferible llenar los pozos con el amortiguador del cátodo sin azul de Coomassie, ya que con el HB no se puede ver la muestra entrando el pozo. Es conveniente usar puntas largas de 200 µl (para geles de secuencia).

Si algunos pozos del gel se quedan sin muestra, llenarlos de "well mix", para tener una corrida homogénea.

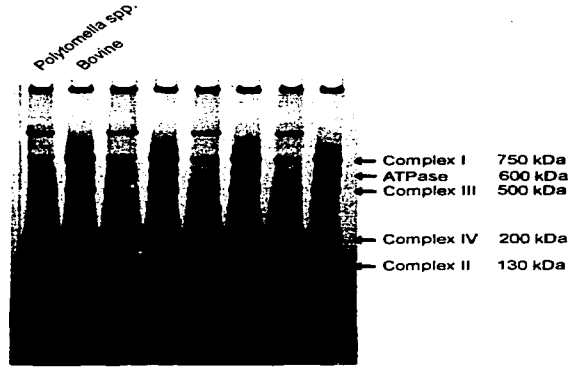
Correr el gel a amperaje constante 15 mA (gel chico) o 25 mA (gel grande). No es necesario precorrer el gel.

La corrida dura approximadamente entre 1 a 1.5 horas para los sistemas chicos y hasta 5 horas para los sistemas grandes. Para mejorar la visibilidad de los complejos proteicos, es recomendable cambiar el amortiguador del cátodo después de 1/3 de la corrida por el mismo amortiguador pero conteniendo únicamente 1/10 de Azul de Coomassie Serva G (amortiguador del cátodo II). Correr hasta que el frente azul llegue a salir del gel o hasta que se halla logrado la separación deseada.

Notas:

- Las proteínas o los complejos proteicos debería verse como bandas bien definidas.
- Puede haber proteínas en el frente de corrida azul.

Ejemplo de BN-PAGE



1,3,5,7 : Mitocondrias enteras de *Polytomella* sp. (2% dodecil-maltósido)
2,4,6,8: Mitocondrias enteras de bovino (1% dodecil-maltósido)

1.4. Geles de segunda dimensión SDS-PAGE

La segunda dimensión desnaturaliza los complejos de la primera dimensión y separa las subunidades de los complejos de acuerdo con su tamaño. Un carril entero de la primera dimensión se pone horizontalmente arriba de un gel desnaturalizante y se corre normalmente.

Para los geles 2D, hay dos métodos disponibles: el gel desnaturalizante tipo Laemmli (Laemmli, 1970) que usa glicina, or un gel conocido como gel de Schägger y von Jagow (1987), que usa tricina en lugar de glicina. Los geles con tricina permiten una resolución de bandas definidas y una mejor separación de las proteínas pequeñas, mientras que los geles tipo Laemmli son más adecuados para resolver proteínas de mayor masa molecular.

Recomendaciones Prácticas:

- Cortar un carril de un gel azul nativo e incubarlo por 30 min en amortiguador del cátodo (1x) con SDS al 1 % y β-mercaptoetanol al 1% en agitación constante.
- Lavar el carril con el amortiguador del cátodo (5 x 1 min) para eliminar el β-mercaptoetanol residual, cuya presencia podría inhibir la polimerización de la acrilamida.
- Poner el carril arriba del vidrio más pequeño y ensamblar las placas. Alternativamente, ensamblar el 'sandwich' de placas sin apretar. insertar el gel de la primera dimensión entre los vidrios y por fin apretar y poner el sandwich verticalmente.
- Poner la solución para el gel separador escurriéndolo a un lado del gel de la primera dimensión, manteniendo el sandwich de placas inclinado para no atrapar burbujas de aire. Usar un gel concentrador de 2 cm de alto para geles grandes y de 1 cm para geles chicos.
- Correr el gel a 50 V hasta que el frente azul haya penetrado el gel concentrador y después aumentar a 100 V.
- Teñir el gel con azul de Coomassie (R250) o con plata.

En lugar del análisis de segunda dimensión de un gel de primera dimensión entero, se pueden analizar complejos específicos resueltos por geles azules. Para esto, se corta el pedazo de gel que contiene el complejo de interés. El pedazo de gel se pone horizontalemente arriba de un gel desnaturalizante; si se requiere un gran cantidad de subunidad (para secuenciación por ejemplo), se pueden apilar los pedazos en el pozo. Es posible hacer pilas de hasta 2 cm, si se usan gel tipo Laemmli para resolver las proteínas.

1.4.1. Geles de tricina-SDS-PAGE

	Geles grandes				Minigeles			
	Acrilamida (ml)			Stacking	Acrilamida (ml)			Stacking
	10%	12%	15%	(ml) (4%)	10%	12%	15%	(ml) (4%)
48.5 % acrilamida	7.4	8.9	11.1	1.0	1.9	2.2	2.8	0.25
1.5 % bis-acrilamida								
Amortiguador del gel 3x		12.0		4.0		3.0		1.0
glicerol 80%		7.2		-		1.8		-
agua distilada	9.4	7.9	5.7	7.0	2.3	2.0	1.7	1.75
TEMED		25 µl		25 µl		6 µl		6 µl
APS (10 %)		150 µl		100 µl		40 µl		20 µl
		36 ml		12 ml		9 ml		3 ml

SOLUCIONES

Solución madre de acrilamida **(100 ml)**

Acrilamida	48.5 %	48.5	g
Bis-acrilamida	1.5 %	1.5	g

Amortiguador para el gel 3 x **(100 ml)**

Tris	3 M	36.33	g
SDS	0.3 %	0.3	g

Disolver en 95 ml de agua destilada
Ajustar el pH a 8.45 con HCl. El HCl ayuda la disolución.

Amortiguador del cátodo (10 x) **(500 ml)**

Tris	1 M	60.57	g
Tricina	1 M	89.6	g
SDS	1 %	5.0	g

No es necesario ajustar el pH

Amortiguador del ánodo (10 x) **(500 ml)**

Tris	1 M	60.57	g

Ajustar el pH a 8.9 con HCl

1.4.2. Geles de glicina-SDS-PAGE

	Geles grandes				Minigeles			
	Acrilamida			Stacking	Acrilamida			Stacking
	10%	12%	14%	(4%)	10%	12%	14%	(4%)
30 % acrilamida 0.8 % bis-acrilamida	12.0	14.4	16.8	1.6	3.0	3.6	4.2	0.4
Tris 3 M pH 8.8		4.5		-		1.13		-
Tris 0.5 M pH 6.8		-		3.0		-		0.75
SDS 20%		100 µl		-		25 µl		-
Agua destilada	19.5	17.1	14.7	7.4	4.8	4.2	3.6	1.85
TEMED		25 µl		25 µl		6 µl		6 µl
APS (10 %)		150 µl		100 µl		40 µl		20 µl
		36 ml		12 ml		9 ml		3 ml

SOLUCIONES

Solución madre de acrilamida **(100 ml)**

Acrilamida	30.0 %	30.0	g
Bis-acrilamida	0.8 %	0.8	g

Los amortiguadores son los reportados por Laemmli (Laemmli, 1970)

2. SOBRE-EXPRESIÓN Y PURIFICACIÓN DE PROTEÍNAS CON 6X HIS-TAGS

Para la sobre-expresión de proteínas con 6x His-tags en *Escherichia coli* y su purificación, se usó el protocolo descrito en la quinta edición del QIAexpressionist de Qiagen.

2.1. Construcción de plásmidos y sobre-expresion de proteínas

En ese trabajo, los vectores de sobre-expresión (OE) usados fueron los vectores pQE30 y pQE60 que tienen la extensión de histidinas 6x en el extremo N-terminal y C-terminal, respectivamente. Se siguen los siguientes pasos:

- Escoger dos enzimas de restricción que no corten en el gen que se quiere clonar pero que corten en el MCS (multiple cloning site) del vector de OE. Esto para asegurar la inserción unidireccional en el plásmido.
- Diseñar cebadores que incluyan los sitios de restricción requeridos (ver el ejemplo más adelante), amplificar el gen por PCR, cortar el gen con las enzimas escogidas, y clonar el DNA en el vector OE cortado con las mismas enzimas.

  Un ejemplo de diseño de los oligodesoxinucleótidos:

  Oligo forward (sentido): 5'-GAC GAGCTC CTG CTG TCG CCG CGC AC-3'
  OH sitio *SacI* cebador

  Oligo reverse (contrasentido): 5'-CTG AAGCTT GGG CAG CTG GCT GGC GC-3'
  OH sitio *HindIII* cebador

  OH = overhang, secuencia adicional para asegurar el corte por las enzima de restricción.

- Transformar las células de *E. coli* competentes (en este trabajo se uso la cepa XL1 Blue MRF').

- Corroborar que existan transformantes.
  Nota: con el vector pQE no se puede hacer el escrutamiento de colonias azules/blancas de los transformantes. Sin embargo, se puede hacer una preparación del plásmido y después un análisis de restricción, o también se puede hacer una reacción de PCR con los transformantes usando los mismos cebadores que se usaron para amplificar el gen. Una reacción de PCR con células transformadas se hace de la manera siguiente: se hierve una cantidad muy pequeña de células resuspendidas en amortiguador TE (50 µl) por 15 min, y se toma 1 µl de la suspensión como templado para la reacción de PCR (20 µl).

- Crecer unos cultivos de 5 ml en medio LB con ampicilina en tubos Falcon de 15 ml a 37°C usando cada transformante positiva, agregar IPTG 1 mM cuando el cultivo llega a una OD_{600} = 0.3-0.6 Guardar 0.5 ml del cultivo (t=0), y dejar crecer el sobrante por 4 horas más.

- Después de 4 horas, tomar 0.2 ml de cultivo (t=4). Centrifugar ambas muestras t=0 y t=4. Resuspender las pastillas en 50 µl del amortiguador de carga 1x para SDS-PAGE, hervir, y correr 10-25 µl de la muestra, dependiendo del tamaño del gel.

- Después de teñir el gel con azul de Coomassie, debería haber una banda abundante del tamaño de la proteína expresada en la muestra t=4. La intensidad de la banda dependerá de la proteína estudiada.

- Para corroborar la presencia de la extensión de histidinas, es recomendable hacer un análisis de tipo Western usando un anticuerpo específico dirigido contra dicha extensión.

Si la proteína sobre-expresada no es visible en el gel y/o si no se obtiene una señal en el Western, lo que se puede/debe hacer es lo siguiente:

1. Secuenciar el plásmido para asegurarse que la construcción es correcta: el 6x His tag tiene que estar en fase con el gen que se clonó.
2. Cambiar las condiciones de sobre-expresión, por ejemplo usar "*E. coli* starter cultures", sobre-expresar a bajas temperaturas (por ejemplo a 25°C), bajar la concentración de IPTG, usar el plásmido pREP4 u otro plásmido supresor para reprimir la sobre-expresión de la proteína hasta que se agregue el IPTG.
3. Cambiar de cepa de *E. coli*.
4. Cambiar de vector de sobre-expresión.

2.2. Purificación de proteínas por afinidad de Ni-NTA

Es recomendable hacer una prueba para ver si la proteína sobreexpresada interactúa con la columna de níquel. A este respecto, es útil llevar a cabo un análisis de tipo Western con un anticuerpo contra la extensión de His, así no se pierde tiempo intentando purificar proteínas que no contienen la extensión de His o que tienen dicha extensión inaccesible.

Indicaciones prácticas:

- La localización de la proteína sobreexpresada se puede hacer por análisis en geles desnaturalizantes y tinción con azul de Coomassie si el nivel de expresión es alto, sino por análisis de tipo Western usando un anticuerpo anti-extensión de His.

1. Dependiendo del nivel de expresión de la proteína, se escoge un volumen de cultivo para obtener la cantidad requerida. Se cosechan las células, y se lava la pastilla en 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, pH 8.0.
2. Resuspender la pastilla en amortiguador de lavado (1/10 del volumen del cultivo inicial) e incubar las células en presencia de 1 mg lisozima/ml, durante 1 hora en hielo.
3. Sonicar la suspensión de células en presencia de 0.1 mM PMSF. Normalmente, la suspensión se vuelve menos turbia. Se puede mejorar el rompimiento de las células aumentando la concentración de lisozima (hasta 5 mg/ml) y/o diluyendo la suspension de células.
4. Centrifugar a 8000 rpm en un rotor SS34; la formación de una pastilla blanca grande (pastilla 1), normalmente indica la presencia de cuerpos de inclusión.
5. Transferir el sobrenadante a tubos de ultracentrifugación y centrifugar a 140,000 x *g* durante 1 hora; la pastilla (pastilla 2) constituye la fracción membranal y el sobrenadante, la fracción soluble.
6. Resuspender las pastillas 1 y 2 en un amortiguador con urea (amortiguador de lavado conteniendo urea 8 M). Si las pastillas no se disuelvan con urea, se puede utilizar clorhidrato de guanidina 6 M. En ese caso, antes de cargar la muestra en SDS-PAGE, se tendrá que precipitar la muestra con TCA o metanol-cloroformo (vea abajo), ya que el clorhidrato de guanidina se precipita en presencia de SDS.
7. Para determinar en qué fracción se encuentra la proteína sobre-expresada, se corre en geles de SDS-PAGE cantidades equivalentes de las proteínas de las pastillas 1, 2 y de la fracción soluble. Se tiñe el gel o se transfieren las proteínas a una

membrana de nitrocelulosa para después hacer un análisis de tipo Western con un anticuerpo anti-extensión de His o con un anticuerpo específico contra la proteína de interés.

- Ya sea que la proteína se encuentre en la fracción soluble o en la fracción membranal, se debería poder purificar la proteína en su estado nativo. Sin embargo, si la extensión de His esta escondida dentro de la proteína no se podrá usar una columna NiNTA.

- Se tendrán que usar detergentes para las proteínas localizadas en las membranas. Las proteínas en cuerpos de inclusión se disolverán en urea o clorhidrato de guanidina, y la purificación se hará en condiciones desnaturalizantes.

- Para producir anticuerpos, es mejor purificar la proteína sobreexpresada en condiciones desnaturalizantes. En ese caso, células enteras de *E. coli* se pueden resuspender directamente en urea o en clorhidrato de guanidina, para después proceder como se describe más adelante. Sin embargo, si la proteína de interés esta localizada en los cuerpos de inclusión será más fácil empezar la purificación a partir de la pastilla 1.

- Estrategia para purificar una proteína sobre-expresada en condiciones desnaturalizantes usando la resina de níquel (Ni-NTA):
  1. Establecer si la proteína (de los cuerpos de inclusión o de las membranas) es soluble en 8 M urea; sino usar clorhidrato de guanidina hasta 6 M.
  2. Equilibrar la columna con el amortiguador de lisis (vea abajo).
  3. Poner la tapa de abajo y después la de arriba, incubar la columna con el lisado por una hora con agitación. También se puede incubar en por ejemplo un tubo.
  4. Abrir la columna y llevar a cabo lavados con el amortiguador de lisis ajustado a pH 6.0, 5.7, 5.3 y 3.0.
  5. Analizar las fracciones que eluyen de la columna en un gel SDS-PAGE.

  Cuando las muestras no contienen clorhidrato de guanidina, se pueden cargar directamente al gel SDS-PAGE. Si no, se tendrá que precipitar las proteínas para quitar la guanidina (vea abajo).

- Para purificar en condiciones nativas una proteína sobreexpresada, seguir el manual de Qiagen. Para purificar la proteína en su estado nativo, se eluye con concentraciones crecientes de imidazol. Para purificar en condiciones desnaturalizantes, se utiliza bajo pH para eluir.

2.3. Precipitación con metanol/cloroformo

La precipitación con metanol/cloroformo es rápida y conveniente cuando el volumen de la muestra es pequeño o cuando la muestra contiene pigmentos (i.e. clorofila).

- Diluir la muestra en 100 µl H_2O
- Agregar 100 µl de cloroformo y 400 µl de metanol
- Agitar en el Vortex
- Agregar 300 µl de H_2O
- Agitar en el Vortex y centrifugar a máxima velocidad (microfuga Eppendorf) durante 5 min.
- Pipetear la fase de arriba (agua/metanol); las proteínas se deben encuentrar en la interfase.
- Agregar 300 µl de metanol, vortexear y centrifugar 5 min. Descartar el sobrenadante.
- Secar la pastilla; disolver la pastilla en 1x amortiguador de carga y hervir.

2.4. Precipitación con TCA

Las proteínas purificadas y desnaturalizadas se pueden concentrar por precipitación. Sin embargo, el clorhidrtao de guanidina también precipita con el TCA.

- Antes de precipitar con TCA, diluir la muestra 3 veces con agua.
- Agregar 100% TCA hasta una concentración final del 10%. Vortexear.
- Dejar la muestra en hielo por 30 min, vortexear cada 10 min.
- Centrifugar 10 min a 14,000 en un rotor SS34 o en una microfuga.
- Agregar 6 volúmenes de agua a la pastilla, vortexear. La guanidina se disolverá mientras que las proteínas se mantendrán precipitadas.
- Centrifugar, agregar etanol (96%) frío a la pastilla, y vortexear.
- Centrifugar. Las proteínas se encuentran en la pastilla.

2.5. Amortiguador de lisis para purificar la proteína sobre-expresada a partir de células enteras de *E. coli*

El amortiguador contiene urea o clorhidrato de guanidina. La urea es preferible por su fácil uso, pero a veces es necesario usar guanidina.

Urea ó:	8	M
Clorhidrato de guanidina	5.5	M
NaH_2PO_4	100	mM
Tris	10	mM
NaCl	500	mM
Glicerol	5	%
Triton X-100	0.5	%
Imidazol	20	mM
β-mercaptoetanol	1	%

Preparar por ejemplo 100 ml de amortiguador de lisis. Repartir en alícuotas de 20 ml y ajustar el pH a 8.0, 6.1, 5.7, 5.3, 3.0.

Generalmente, las células enteras de *E. coli* se disuelven bien en este amortiguador. Resuspender las células en 1/10 del volumen original del cultivo, incubar 1 hora a temperatura ambiente, vortexeando de vez en cuando. El sobrenadante resultante de la centrifugación de la suspensión está listo para la purificación en columnas de níquel.

2.6. Amortiguador de lisis para purificar proteínas sobre-expresadas presentes en cuerpos de inclusión

Cuando se tienen cuerpos de inclusión, los componentes siguientes son normalmente suficientes:

Urea ó:	8	M
Guanidina HCl	6	M
NaH_2PO_4	100	mM
Tris	10	mM
NaCl	500	mM

Puede ser útil emplear bajas cantidades de β-mercaptoetanol (por ejemplo 0.1 %). Continuar como se indicó para el amortiguador de lisis para células enteras de *E. coli*.

APÉNDICE III

ARTÍCULOS PUBLICADOS Y SOMETIDOS A PUBLICACIÓN

ARTÍCULO II

***Polytomella* sp. growth on ethanol.**
Extracellular pH affects the accumulation of mitochondrial cytochrome c_{550}.

Atteia, A., van Lis, R., Ramírez, J. and González-Halphen, D. (2000)

Eur J Biochem 267, 2850-2858

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Polytomella spp. growth on ethanol

Extracellular pH affects the accumulation of mitochondrial cytochrome c_{550}

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A defined medium with ethanol as sole carbon source was devised for growth of the colorless, unicellular alga *Polytomella* spp. Cell density on this carbon source was related to extracellular pH. An acidic pH was required for ethanol utilization; best yields were obtained at pH 3.7. Spectroscopic analysis of the cells showed that the concentration of cytochrome *c* per cell was 40% higher than at pH 6.0; the concentrations of cytochrome a_{606} (cytochrome *c* oxidase) and b_{566} (cytochrome bc_1 complex) were the same. A soluble cytochrome c_{550} was purified from cells grown at pH 3.7 and characterized by peptide sequencing as the 12-kDa cytochrome c_{550} of the mitochondrial respiratory chain. Immunoblots of total cell proteins showed higher accumulation of cytochrome c_{550} at pH 3.7 than at pH 6.0. RNA blot analysis gave clear evidence of the abundance of c_{550} transcript in cells grown at pH 3.7. The amount of mitochondrial proteins obtained from cells grown at pH 3.7 was twofold higher than that of cells grown at pH 6.0. Mitochondria isolated from both cell types readily oxidized succinate, malate or ethanol. The rates of oxygen uptake were 20–25% higher in mitochondria from cells grown at pH 3.7. Cyanide and antimycin A inhibited respiration with succinate up to 95% in both types of mitochondria. The participation of cytochrome c_{550} in mitochondrial electron transport from succinate to oxygen was shown by spectral measurements.

Keywords: *Polytomella* spp.; *Chlamydomonas reinhardtii*; ethanol metabolism; cytochrome *c*; extracellular pH.

Unicellular algae from the genus *Polytomella* are found in various habitats including fresh water ponds, meadow ditches and greenhouse soils [1]; up to now, eight distinct species have been isolated in pure culture. The members of the genus *Polytomella* are nonpigmented, heterotrophic algae that have neither cell walls nor chloroplasts. On the basis of common physiological and morphological features shared with the photosynthetic algae *Chlamydomonas*, the genus *Polytomella* has been assigned to the family of the Chlamydomonaceae [2]. According to Round [3], *Polytomella* arose from a *Chlamydomonas*-like ancestor by the loss of photosynthetic pigments and cell wall. Recent molecular and biochemical data on several key proteins of the mitochondrial respiratory chain and the ATP synthase of the colorless alga *Polytomella* spp. and the photosynthetic alga *Chlamydomonas reinhardtii* have strengthened the hypothesis of Round [4–7].

Earlier studies on *Polytomella caeca* have shown that this alga can grow on different carbon sources, including organic acids and alcohols of different chain length [8,9]. Depending on the carbon source and culture media, *P. caeca* growth can take place in a pH range of 1.4 to pH 9.6 [8,10]. These studies illustrate the remarkable potential of this alga to adapt its metabolism to growth conditions. However, the metabolic pathways and bioenergetics of the algae *Polytomella* have not been further studied.

In this work, we studied the growth of *Polytomella* spp., a strain isolated from green house soils, with a particular reference to the effect of pH. The data revealed previously undescribed features of the bioenergetics of *Polytomella* that concern the profound effect of low extracellular pH on (a) the steady-state accumulation of the mitochondrial soluble cytochrome c_{550} and (b) the mitochondrial respiration in cells grown on ethanol.

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Abbreviation: TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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MATERIALS AND METHODS

Growth conditions

The colorless alga *Polytomella* spp. (198.80, E.G. Pringsheim) was obtained from the alga collection at the University of Göttingen (Germany). The alga was grown aerobically without agitation at room temperature in 2-L flasks containing 1 L of culture medium. All the culture media contained 1 mM potassium phosphate (pH 7.0), 7.4 mM NH_4Cl, 0.3 mM $CaCl_2$, 0.5 mM $MgSO_4$, and the following trace elements: 1.39 μM $ZnSO_4$, 0.8 μM H_3BO_3, 2.65 mM $MnSO_4$, 0.74 μM $FeCl_3$, 0.16 mM $CuSO_4$, 0.83 μM $NaMoO_4$, and 0.6 μM KI. The media used were: TAP medium (Tris base, 20 mM; the pH was adjusted to 7.2–7.3 by adding 1 mL of glacial acetic acid, 17.4 mM acetate) [11]; $TAP_{pH5.2}$ medium (20 mM Tris base, 30 mM sodium acetate; the pH was adjusted to 5.2 with HCl); EP medium (40 mM ethanol; the pH was adjusted to 6.0 with HCl); TEP medium (20 mM Tris base, 40 mM ethanol; the pH was adjusted to 7.8 with KOH); MEP medium [20 mM Mes (pK_a 6.1), 40 mM ethanol; the pH was adjusted to 5.2–5.3 using KOH]; and REP medium (20 mM tartaric acid; pK_a values 2.93 and 4.23), pH was adjusted to 3.7 with HCl]. All the culture media were autoclaved and then filtered ethanol was added to EP, MEP, TEP and REP media.

Prior to cell inoculation, thiamine and cyanocobalamin were added to all growth media to a final concentration of 20 $\mu g \cdot L^{-1}$ and 1 $\mu g \cdot L^{-1}$, respectively [12]. To follow growth, culture media were inoculated to a cell concentration of 0.5–1 × 10^3

cells·mL^{-1}. During growth, samples were taken to determine cell concentration and pH (ΦBranson pH meter). Cell number was determined using a Neubauer cytometer. To immobilize the cells, Lugol's solution (5% iodine, 10% potassium iodide) was added to the sample and when necessary, cells were diluted in 0.3 M sorbitol.

Cell harvesting

Exponentially grown cells were harvested, washed in 0.3 M sorbitol, 10 mM Mes, pH 6.0 (adjusted with KOH) and resuspended in the same buffer, and used immediately for biochemical and spectroscopic studies.

Spectroscopy

Before spectroscopic analysis, freshly harvested cells were sonicated 4 × 15 s, at 40 W (Branson Sonifier 250, Sonic Power Company). Visible spectra were recorded at room temperature with a DW-2a UV/Vis SLM-Aminco spectrophotometer modified with the OLIS DW2 CONVERSION and OLIS software (On-line Instrument System Inc.). Bilateral curved slits with a spectral bandpass of 3 nm were used at a scanning speed of 5.0 nm·s^{-1}. Wavelength calibration was made with purified horse heart cytochrome *c* (Type III; Sigma Chemical Co.). Oxidation and reduction of the cytochromes was achieved by adding, respectively, a few grains of ammonium persulfate and dithionite. The spectra shown below represent an average of five separate batches of cells. The isolated mitochondrial cytochrome *c* was oxidized by adding ferricyanide [13]. Ferricyanide was removed by an overnight dialysis at 4 °C against 50 mM Tris/HCl, pH 8.0. Spectra of the cytochrome c_{550} were recorded before and after reduction with ascorbate. Reduction of mitochondrial cytochromes was achieved by adding 10 mM of succinate, 2.5 mM cyanide and 2.5 μM antimycin A to the mitochondria; oxidation was achieved by adding a few grains of persulfate.

Protein anaiysis

Cells or mitochondria were solubilized in 2% SDS, 12% sucrose after addition of 100 mM dithiothreitol [13] and boiled for 2 min. Polypeptides were separated on 13% acrylamide gels in a Laemmli buffer [14]. Hemes were detected by peroxidase activity of heme binding subunits using TMBZ as described by Thomas *et al.* [15]. Immunodetection was carried out with an ECL kit (Amersham) using antibodies against *C. reinhardtii* cytochrome *c* [13] and the β subunit of the mitochondrial ATPase of *Polytomella* spp. For N-terminal amino-acid determination, the cytochrome *c* (250 pmol) was electrotransferred onto poly(vinylidene difluoride) membranes (Immobilon-P transfer membranes, Millipore) as described in Atteia *et al.* [7]. For internal sequencing, cytochrome *c* separated by SDS/PAGE was subjected to trypsinolysis and HPLC separation. Protein sequencing was carried out by J. d'Alayer at the Laboratoire de Microséquençage des Protéines (Institut Pasteur, Paris, France). Apparent molecular masses were estimated using commercial molecular mass markers (Protein Ladder, Pharmacia, or Benchmark markers, Gibco-BRL). Protein concentrations were determined by the method of Markwell *et al.* [16].

Purification of *Polytomella* spp. mitochondrial cytochrome c_{550}

Cells grown at pH 3.7 were harvested, resuspended in 20 mM potassium phosphate, 150 mM Na_2CO_3, 150 mM dithiothreitol, frozen, thawed and ultracentrifuged for 30min at 160 000 *g* in a 60Ti rotor (Beckman). All steps were carried out at 4 °C. Ammonium sulfate fractionation of the soluble proteins was performed at 40, 65 and 90% saturation. Proteins that precipitated at 90% saturation were resuspended in 50 mM Tris, 5 mM ε-amino-caproic acid, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, pH 7.8 (buffer A) and dialyzed against the same buffer. The dialyzed sample was then subjected to a two-step ion-exchange chromatography; first, proteins were loaded onto an anion-exchange column (DEAE-Biogel, Sigma) equilibrated with buffer A, and those that ran through were then loaded onto a cation-exchange column (Bio-Rex 70, Biorad). Cytochrome *c* was eluted from this column with 100 mM NaCl in buffer A, and concentrated by ultrafiltration with a Centricon-10 (Amicon).

Isolation of mitochondria and oxygen uptake measurements

Polytomella spp. mitochondria were isolated from cells harvested in their log-phase following the protocol used for preparing coupled mitochondria from the green alga *C. reinhardtii* [17], except that the Percoll gradient centrifugation step was omitted. The yield was approximately 4 mg of mitochondrial proteins per 1 g (wet weight) of cells grown at pH 6.0 and of 8–10 mg for cells grown at pH 3.7. Oxygen consumption of freshly prepared mitochondria was measured at 27 °C using a Clark oxygen electrode in a reaction vessel of 3 mL of air-saturated respiration buffer (10 mM potassium phosphate buffer, pH 7.2, 0.1% BSA, 0.25 M sorbitol, 10 mM KCl, 5 mM $MgCl_2$). The respiratory substrates used were succinate (10 mM), malate (10 mM malate, 10 mM glutamate, 1 mM NAD) or ethanol (20 mM ethanol, 1 mM NAD). Measurements were performed with 0.25–0.35 mg of proteins per ml of assay buffer.

RNA isolation and hybridization

Total RNA from *Polytomella* spp. was isolated using Trizol Reagent (Gibco-BRL). RNA samples were separated on 0.22 M formaldehyde agarose gels in Mops/formaldehyde buffer: 20 mM Mops, 40 mM sodium acetate, 8 mM EDTA, pH 7.0 and 0.22 M formaldehyde, and transferred to Hybond N membrane (Amersham). Approximately 10 μg of total RNA was loaded per lane. DNA probes were labelled using the Random Primers DNA labeling system (GibcoBRL) using [α-^{32}P]dCTP (DuPont). Probes were *Cyc* cDNA from *C. reinhardtii* [18], and *TubB1* cDNA from *P. agilis* (now renamed *P. parva*) [19]. Prehybridization and hybridizations were carried out at 42 °C in 6 × NaCl/Cit, 1% SDS, 50% deionized formamide.

Electron microscopy

Isolated mitochondria were fixed in 4% paraformadehyde and 2% glutaraldehyde, postfixed in 1% osmium tetroxyde and embedded in Epon-Araldite resin.

RESULTS

The effect of the extracellular pH on *Polytomella* spp. growth on acetate or ethanol

Our experiments on the characterization of different mitochondrial protein complexes of *Polytomella* spp. [7,20,21] were carried out with cells grown on a rich culture medium containing 30 mM acetate, 0.2% bactotryptone and 0.2% yeast extract. In this medium, the algae grew with a doubling

Table 1. ***Polytomella*** **spp. growth on acetate or ethanol as sole carbon source.** Initial pH values correspond to the values of extracellular pH of a freshly prepared medium after cell inoculation; final pH values, to the values of extracellular pH when the cells have reached stationary phase. The culture media selected to study the effect on extracellular on metabolism and bioenergetics of ethanol-grown *Polytomella* spp. cells are indicated in bold type.

	Culture medium (mM)		Cell concentration (10^5 cells·mL^{-1})	Initial pH	Final pH
TAP medium	Acetate	17	0.3–0.4	7.2–7.3	7.6–7.7
	Tris	20			
$TAP_{pH5.2}$ medium	Acetate	40	1.6–1.8	5.2–5.3	7.4–7.6
	Tris	20			
EP medium	Ethanol	40	2.9–3.2	5.9–6.0	2.2–2.5
TEP medium	Ethanol	40	0.2–0.3	7.2–7.3	6.9–7.0
	Tris	20			
MEP medium	Ethanol	40	2.8–3.0	5.8–6.0	5.4–5.6
	Mes	20			
REP medium	Ethanol	40	7.0–7.2	3.7–4.0	3.3–3.5
	Tartaric acid	20			

time of 10–13 h, reaching a final cell concentration of 1.5–1.6 × 10^5 cells·mL^{-1} (not shown). For our purposes, this medium has not been chemically defined, and therefore could not be used to investigate the carbon metabolism of *Polytomella*. As *Polytomella* and *Chlamydomonas* are related algae, we attempted to grow the colorless alga on TAP medium (20 mM Tris, 17 mM acetate, and 1 mM potassium phosphate, pH 7.2–7.3), a medium commonly used to grow the photosynthetic alga [11]. The cell density obtained on TAP medium was 0.3–0.4 × 10^5 cells·mL^{-1} (Table 1); this is four to five times lower than the cell density obtained on rich medium, and less than 10% of *Chlamydomonas* grown on TAP medium under illumination. The low growth of *Polytomella* on TAP medium was not due to an insufficient carbon source because no further growth was observed with 40 mM acetate. However, the cell densities on TAP medium were significantly increased when the pH of the medium was lowered. The highest cell densities were obtained when initial pH was 5.2 ($TAP_{pH5.2}$ medium); under these conditions, cell densities reached values similar to those obtained with the rich culture medium (Table 1).

In an early study, Wise [8] showed that *P. caeca* could grow on various alcohols as carbon sources and that the highest cell densities were obtained with ethanol. To explore if *Polytomella* spp. could utilize ethanol, we tested its growth on TEP medium in which the acetate of TAP medium was replaced by ethanol (40 mM). In this medium, growth was poor (Table 1). By lowering the pH from 7.2 to 6.0, growth was significantly increased, indicating that acidic pH values are required for ethanol utilization by *Polytomella* spp. The cell density obtained on EP medium (with no additional buffer) was approximately twice that of on acetate medium ($TAP_{pH5.2}$ medium) (Table 1, Fig. 1A). During growth, a marked acidification (up to 3 pH units) of the culture medium was observed (Fig. 1B). These data on the growth of *Polytomella* spp. on EP medium indicated that ethanol uptake and cell division could

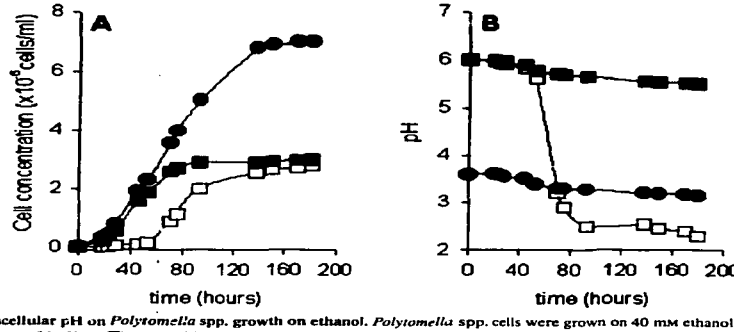

Fig. 1. Effect of extracellular pH on ***Polytomella*** **spp. growth on ethanol.** *Polytomella* spp. cells were grown on 40 mM ethanol at different values of pH in the presence or absence of buffer: (□) no additional buffer (EP medium); (■) 20 mM Mes (MEP medium); (●) 20 mM tartaric acid (REP medium). (A) Growth curves; (B), pH curves. Curves shown are representative of five independent experiments.

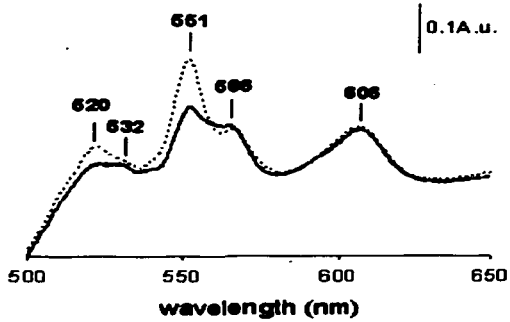

Fig. 2. Difference absorption spectra of *Polytomella* spp. cells grown on ethanol at pH 6.0 (–) and pH 3.7 (···). Cell suspensions were reduced and oxidized with dithionite and persulfate, respectively. Maxima are indicated to the nearest 0.5 nm. The cell concentration used was approximately 2.3×10^7 cells·mL^{-1}.

take place over a wide range of pH values. At pH values below 3.0, the cells started to lose their flagella and undergo sedimentation.

To investigate the effect of pH on the growth of the colorless alga on ethanol, different nonmetabolizable buffers were added to the growth media. Growth of the alga was followed at pH 6.1 (MEP medium) and at pH 3.7 (REP medium). These buffers maintained pH variations below 0.6 pH units. The growth curves in Fig. 1A show that growth increased as the pH was lowered. For example, cell densities at pH 3.7 were about three times higher than at pH 6.0. Under both conditions, the cells were flagellated and distributed homogeneously in the medium.

In the following experiments, we examined cells grown on ethanol at pH 6.0 and pH 3.7 in an attempt to gain insights into the effect of the extracellular pH on the metabolism and bioenergetics of *Polytomella* spp.

Difference spectra of *Polytomella* spp. cells grown on ethanol

The cytochrome contents of cells grown on ethanol at pH 6.0 and pH 3.7 were analyzed by visible spectroscopy. The α region of the dithionite-reduced minus persulfate-oxidized spectra of cells (2.3×10^7 cells·mL^{-1}) exhibited absorption peaks at 551, 566 and 606 nm (Fig. 2), indicating, respectively, the presence of *c*-, *b*-, and *a*-type cytochromes in both batches of cells. The differential spectra of purified mitochondrial cytochrome bc_1 complex and cytochrome *c* oxidase type aa_3 of *Polytomella* spp. have been reported previously. The cytochromes *b* and *a* exhibit α bands with maxima at 566 nm and 606 nm, respectively; these maxima are 4–5 nm red-shifted in comparison to those of plants or mammals [21]. Assuming that the absorption peaks at 566 nm and 606 nm of the spectra in Fig. 2 reflect the presence of mitochondrial cytochromes *b* (bc_1 complex) and *a* (cytochrome *c* oxidase), the results indicate that the pH of the ethanol-containing medium did not affect their level of accumulation in *Polytomella* spp. In contrast, the level of accumulation of *c*-type cytochromes (α band at 551 nm; β band at 519 nm) is clearly influenced by the extracellular pH. The calculated A_{566}/A_{551} ratios [21] for pH 6.0 and cells grown at pH 3.7 was 0.73 and 0.40, respectively. The pelleted cells also indicated a difference in cytochrome content; the color of cells grown at pH 3.7 was pink, whereas that of cells grown at pH 6.0 was brown.

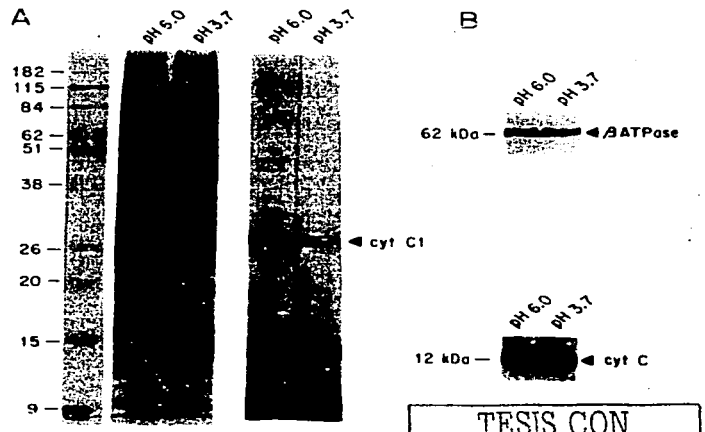

Fig. 3. Accumulation of *Polytomella* spp. mitochondrial cytochrome c_{550}. (A) Whole cell polypeptides were separated on SDS/PAGE (13% acrylamide) and visualized by Coomassie blue (left panel, 2×10^5 cells) or TMBZ (right panel, 4×10^5 cells; approximately 200 μg of proteins). *Polytomella* spp. cytochrome c_1 is indicated as cyt c_1. Molecular mass markers were Benchmark Markers (Gibco-BRL). (B) Immunological reactions. Whole cell polypeptides (200 μg of proteins) were separated on 13% acrylamide SDS/PAGE, blotted on a nitrocellulose membrane and probed with specific antibodies for cytochrome mitochondrial c_{550} (lower blot) and the β subunit of mitochondrial ATPase (upper blot).

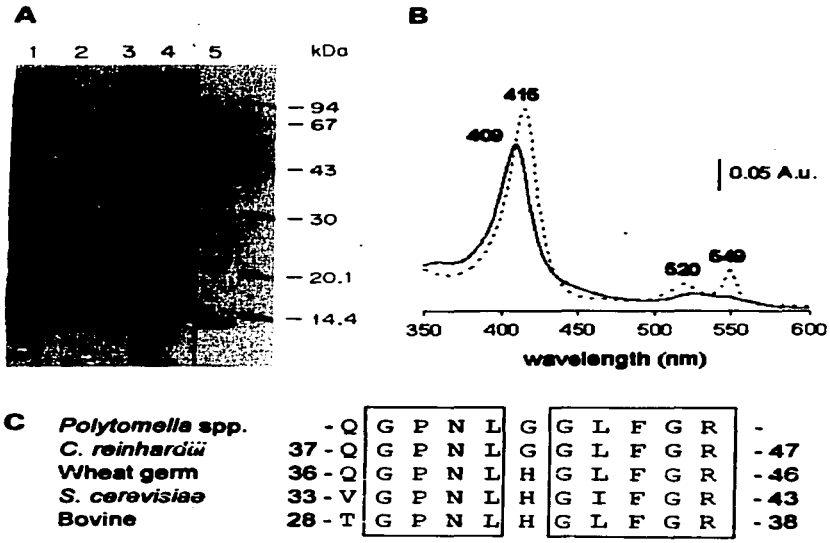

Fig. 4. ***Polytomella*** **mitochondrial cytochrome** c_{550}. (A) Purification of the mitochondrial c_{550} from cells grown at pH 3.7. Lane 1, whole cell polypeptides; lane 2, soluble proteins; lane 3, proteins precipitated with 90% saturation of ammonium sulfate; lane 4, fraction of proteins not retained on anion-exchange column; lane 5, fraction of proteins eluted with 100 mM NaCl from cation-exchange column. Molecular mass markers were from Pharmacia. (B) Absorption spectra of cytochrome c_{550}. Oxidized and reduced spectra of the purified 12-kDa cytochrome. The cytochrome was oxidized by ferricyanide. Ferricyanide was removed by dialysis. Spectra were recorded at room temperature in 50 mM Tris/HCl, pH 8.0. Before (–) and after (···) reduction by few grains of ascorbate. (C) Amino-acid sequence of an internal fragment of *Polytomella* spp. mitochondrial cytochrome c_{550}. The amino-acid sequence of an internal fragment of *Polytomella* spp. mitochondrial cytochrome c_{550} obtained by trypsinolysis is compared with the corresponding fragments of mitochondrial cytochrome c_{550} from other eukaryotes. The sequences used for comparison are those of *C. reinhardtii* [18]; wheat [22]; *S. cerevisiae* [23]; and bovine [24].

Steady-state mitochondrial cytochrome c_{550} accumulation is enhanced at pH 3.7

To assess the nature of the cytochrome(s) *c* whose accumulation depended on the pH of the medium, whole cell polypeptides were separated by SDS/PAGE stained with TMBZ to detect hemes [15] or with Coomassie-Blue for total proteins. Coomassie-blue stained SDS/PAGE (Fig. 3A, left panel) revealed some differences in the protein pattern between cells grown at different pH values, with apparently more high molecular mass proteins and less low molecular mass proteins at pH 6.0 than at pH 3.7. The heme-stained polypeptide pattern of cells grown at pH 3.7 and pH 6.0 was qualitatively the same, exhibiting two major bands (Fig. 3A, right panel). One of these bands was a 30-kDa polypeptide that was previously identified as the membrane-bound cytochrome c_1 [20]. The other band was a low molecular mass cytochrome (12 kDa), so far uncharacterized. For a similar number of cells (4×10^5 cells), the amount of heme-stained low molecular mass cytochrome was significantly higher in cells grown at pH 3.7 than at pH 6.0. This 12-kDa cytochrome cross-reacted with an antibody raised against the mitochondrial cytochrome c_{550} of the related photosynthetic alga *C. reinhardtii*. The immunoblot analysis demonstrated that the level of accumulation of this cytochrome was highly enhanced in the cells grown at pH 3.7 (Fig. 3B). In contrast, the level of accumulation of the β subunit of the mitochondrial ATPase was not influenced by the pH of the external medium (Fig. 3B).

The low molecular mass cytochrome was purified from cells grown at pH 3.7. The purification procedure consisted of: (a) a fractionation of soluble and membrane proteins; (b) ammonium sulfate precipitation steps at 40, 65 and 90% saturation; and (c) a combination of anion- and cation-exchange chromatographies (Fig. 4A). This isolation procedure (see Materials and methods) indicated that the 12-kDa cytochrome is a soluble, basic protein. The spectrum of the ascorbate-reduced form characterized by absorption peaks at 415, 520 and 550 nm, is typical of a *c*-type cytochrome (Fig. 4B). The reduction by ascorbate indicated a redox potential more positive than +60 mV. Attempts to determine the N-terminal sequence of this polypeptide were

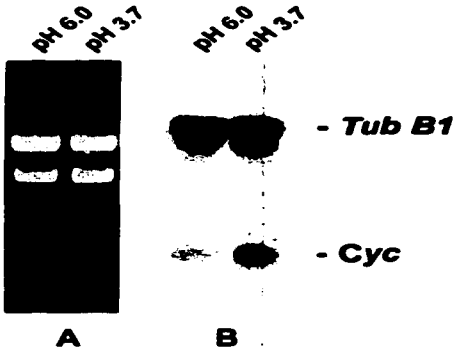

Fig. 5. RNA blot analysis of *Cyc* mRNA levels. Total RNA was isolated from *Polytomella* spp. cells grown on ethanol at pH 6.0 or pH 3.7 and hybridized as described in Materials and methods. The same blot was first probed for *Cyc* mRNA and then for *TubB1* mRNA. The *TubB1* transcript was used as an internal control for loading equivalent amounts of RNA. (A) agarose gel; (B) RNA blot.

unsuccessful, suggesting a blocked N-terminus. Therefore, the protein was subjected to trypsinolysis followed by HPLC separation of the generated fragments. The amino-acid sequence obtained for one of these peptides was compared to the corresponding sequences of mitochondrial c_{550}. The sequence of 11 residues was identical to that of the sequence of the corresponding fragment in the cytochrome *c* from *C. reinhardtii*, and homologous to the sequences of mitochondrial cytochrome c_{550} from various sources (Fig. 4C). Therefore, our spectroscopic, biochemical, and immunochemical data indicated that the pH of the ethanol-containing medium regulated the accumulation of the mitochondrial cytochrome c_{550}.

Polytomella spp. *Cyc* mRNA levels are increased at pH 3.7

Total RNA was isolated from *Polytomella* spp. cells grown at pH 6.0 and pH 3.7 and subjected to RNA blot analysis using specific probes for the cytochrome c_{550} gene (*Cyc*) and the gene encoding β-tubulin (*TubB1*). The *TubB1* gene was used as an internal control for loading equivalent amounts of RNA in the gels. At pH 3.7 and 6.0, we observed expression of *Cyc* gene (Fig. 5). However, *Cyc* mRNA steady-state levels were significantly higher at pH 3.7.

Ultrastructure of mitochondria isolated from *Polytomella* spp. cells grown on ethanol

Polytomella mitochondria were isolated according to the protocol described by Eriksson *et al.* [17] for the preparation of *C. reinhardtii* mitochondria. Surprisingly, the yield of the mitochondrial preparation was related to the pH of the culture medium. Approximately 9 and 4 mg of mitochondrial proteins could be obtained per gram of cells (wet weight) grown at pH 3.7 and at pH 6.0, respectively. Electron microscope

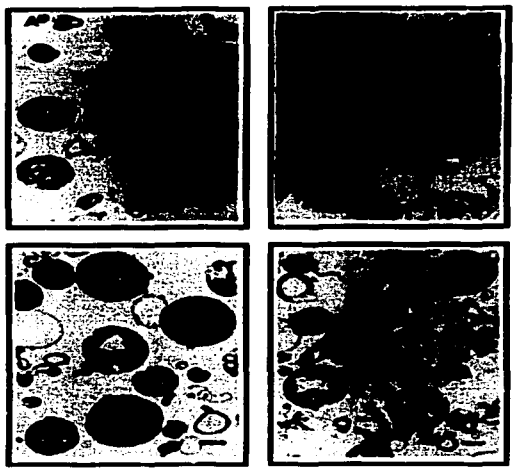

Fig. 6. Electron micrographs of mitochondria isolated from *Polytomella* spp. cells. Mitochondria were isolated from *Polytomella* spp. cells grown on ethanol at pH 6.0 (A,B); at pH 3.7 (C,D). Bars represent 0.5 μm.

examination of the preparations of mitochondria from cells grown at pH 6.0 and pH 3.7 revealed that the fractions consisted of mitochondria comparable in size and shape (Fig. 6). However, it was observed that the mitochondria from cells grown at pH 3.7 appeared slightly more broken than those grown at pH 6.0.

Structural and functional studies on mitochondria isolated from *Polytomella* cells grown at pH 3.7 and 6.0

The fact that the level of accumulation of the cytochrome c_{550} was influenced by the extracellular pH prompted us to investigate the structure and function of the mitochondria isolated from *Polytomella* spp. cells grown at pH 6.0 and

Table 2. Oxygen uptake by isolated mitochondria. Oxygen uptake of mitochondria from cells grown at pH 6.0 and at pH 3.7 on different substrates. Oxygen uptake rates are given in nmol O_2 $min^{-1} \cdot mg^{-1}$ proteins. Standard error is shown in parentheses. Values are given as least squares means of 4–5 experiments ($F = 17.0402$, $P < 0.0001$).

	pH 6.0	pH 3.7
Succinate	74.8 (1.977)	93.0 (1.977)
+ 1 mM KCN	4.0 (1.977)	4.2 (1.977)
+ 1 mM Antimycin A	3.5 (1.977)	4.0 (1.977)
Malate	63.0 (2.210)	76.8 (1.977)
Ethanol	80.0 (1.977)	107.8 (2.210)

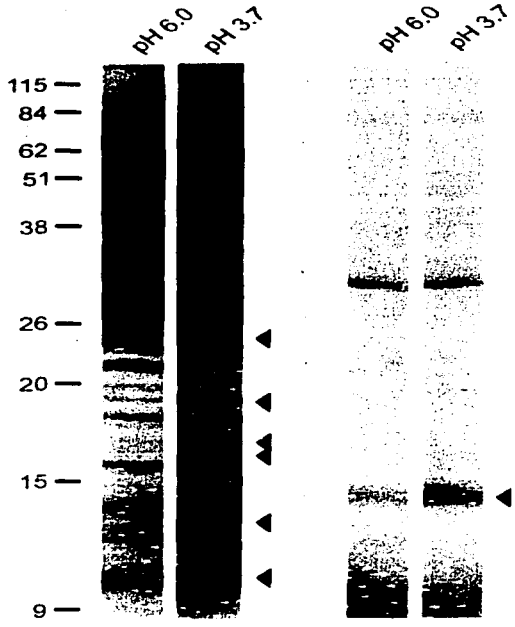

Fig. 7. Accumulation of *Polytomella* spp. mitochondrial cytochrome c_{550} and small molecular mass polypeptides. Polypeptides from isolated mitochondria were separated on SDS/PAGE (14% acrylamide) and visualized by Coomassie blue (200 μg of proteins, left panel) or TMBZ (300 μg of proteins, right panel). The small molecular mass polypeptides that are more abundant at pH 3.7 are indicated with arrows. Molecular mass markers were Benchmark Markers (Gibco-BRL).

pH 3.7. The polypeptide patterns of mitochondria isolated from cells grown at two different pH values were explored. Coomassie blue and TMBZ stained gels revealed differences in the polypeptide pattern between mitochondria isolated from cells grown at different pH values, as shown in Fig. 7. Besides cytochrome c_{550}, several low molecular mass polypeptides were found to be more abundant at pH 3.7.

Mitochondria prepared from both sources readily oxidized succinate, malate and ethanol. With all the substrates, the rate of oxygen consumption was 20–25% higher in mitochondria isolated from cells grown at pH 3.7 than in mitochondria from those grown at pH 6.0 (Table 2). The respiration on ethanol was optimal when NAD was added, suggesting that *Polytomella* spp. has a mitochondrial NAD+-linked alcohol dehydrogenase.

Respiration with succinate by the two mitochondrial preparations was sensitive (up to 95%) to 1 mM of cyanide or 1 μM antimycin A (Table 2). The I_{50} for potassium cyanide, determined with a Dixon plot, was 25–30 μM for both preparations (not shown). Using spectroscopy, we explored the involvement of the cytochrome c_{550} in the electron pathway from succinate to oxygen in both preparations of mitochondria. Difference spectra were recorded after incubation of the mitochondria with 10 mM succinate, 2 mM cyanide, and 2 μM antimycin A. It is noted that electron-transfer within *Polytomella* spp. cytochrome bc_1 complex is fully inhibited by 1 μM of antimycin A [21]. The succinate-reduced vs. persulfate-oxidized spectra of mitochondria isolated from pH 6.0 and pH 3.7 grown cells are shown in Fig. 8. The A_{566}/A_{551} ratios were of 0.85 for the mitochondria isolated from cells grown at pH 6.0 and 0.52 for those isolated from those grown at pH 3.7. These ratios were similar to those obtained with whole cells, therefore indicating a mitochondrial location of the soluble cytochrome c_{550} and its participation in the electron transport chain.

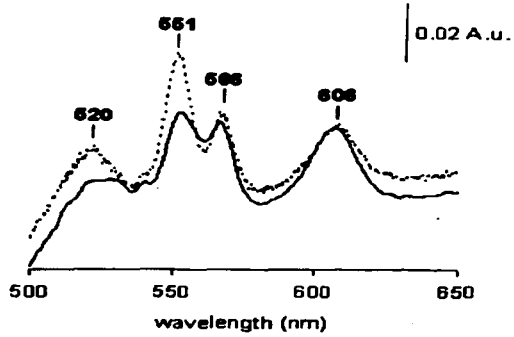

Fig. 8. Succinate reduced vs. persulfate oxidized mitochondria. Protein concentration was 8 mg·mL^{-1}. To the samples reduced with 10 mM succinate, 2.5 μM antimycin A and 2.5 mM cyanide were added. (–) Mitochondria isolated from cells grown at pH 6.0. (—) Mitochondria isolated from cells grown at pH 3.7.

DISCUSSION

Metabolic flexibility of *Polytomella* spp

In the study of the growth of *Polytomella* spp. the first step was to define a culture medium in which carbon, nitrogen and phosphate concentrations could be controlled. Satisfactory growth was obtained on a medium derived from the chemically defined TAP medium used for growth of the photosynthetic alga *C. reinhardtii*, if the initial pH was below 7.0. We found that *Polytomella* spp. grew much better at acidic pH on either ethanol and acetate which makes this alga an acidophilic organism. Our studies on the physiology of the heterotrophic alga *Polytomella* spp. revealed some marked differences with the evolutionary-related alga *Chlamydomonas*: (a) *Polytomella* spp., unlike *C. reinhardtii*, grows poorly on acetate at pH values near neutrality, and (b) *Polytomella* spp. can utilize ethanol as a sole carbon source, a substrate that is very poorly

utilized by *C. reinhardtii* (A. Atteia and R. van Lis, unpublished data). These differences may be due to the absence of photosynthetic activity in the colorless alga. However, assuming that *Polytomella* species have evolved from a *Chlamydomonas*-like ancestor [3], our data suggest that in addition to loss of its functional photosynthetic apparatus, *Polytomella* has acquired new metabolic pathways.

All the physiological studies carried out on the algae of the genus *Polytomella* (see above) [8–10] indicate that these algae can cope with a wide variety of environmental conditions and therefore make *Polytomella* useful to investigate the mechanisms used by eukaryotes to adapt to their environment.

Ethanol metabolism of the alga

As shown above, *Polytomella* spp. can derive energy for growth and cell maintenance from ethanol under acidic conditions. In the absence of buffer in an ethanol-containing medium (EP medium), the growth of *Polytomella* spp. caused an acidification of the extracellular medium. This acidification is not a consequence of the uptake of ethanol, as this molecule can permeate the cells by simple diffusion without involvement of any carrier. The extracellular acidification observed is likely to be a consequence of the oxidation of ethanol as shown by Loureiro-Dias and Santos [25] for *S. cerevisiae*. By means of NMR techniques, the authors showed that a product of the oxidation of ethanol, acetic acid, accounts for the decrease of the intracellular pH. In order to maintain pH homeostasis in the cells, protons are expelled from the cytoplasm to the extracellular medium by a plasma membrane H^+-ATPase.

The significant acidification of the culture medium by *Polytomella* spp. could be prevented by addition of buffer (tartaric acid or Mes). A medium with tartaric acid as buffer (REP medium) allowed better growth than an unbuffered medium (EP medium), suggesting that growth is inhibited at very low pH (pH 2.0–2.5). An external acidification of *S. cerevisiae* cultures (from pH 6.5 to pH 3.5) has been shown by means of ^{31}P NMR to cause a decrease in cytoplasmic pH [26]. In cells grown at low pH, the amount of the plasma membrane H^+-ATPase was higher than in cells grown at pH 6.0 [27]. Further investigation on *Polytomella* spp. will be needed to determine whether extracellular pH influences the intracellular pH and to characterize the mechanism(s) used by the alga grown at pH 3.7 to sustain a large transmembrane pH gradient.

The steady-state levels of *Cyc* mRNA and cytochrome c_{550} are regulated by extracellular pH

Analysis of SDS/PAGE stained with Coomassie blue or TMBZ of whole cells showed relatively more low molecular mass proteins at pH 3.7 and high molecular mass proteins at pH 6.0. Interestingly, similar polypeptide patterns were observed with isolated mitochondria, suggesting a regulation of mitochondrial protein accumulation in *Polytomella* spp. by extracellular pH. It has been reported that for a number of bacteria, extracellular pH affects the level of accumulation of distinct groups of proteins. In *Escherichia coli*, the acid shock proteins expressed after transfer of the cells from pH 6.9 to pH 4.3 represent stress proteins induced by osmolarity, aerobiosis or low temperature [28]. In the acidophilic bacterium *Thiobacillus ferroxidans*, the synthesis of several cellular components, such as the 36-kDa porin-like protein, increases in very acidic conditions [29].

In this work, we focused on one of the low molecular polypeptides of *Polytomella* spp., a 12-kDa cytochrome, whose accumulation varies with extracellular pH. Using spectroscopy, immunoblot analysis, and amino-acid sequencing, we identified this polypeptide as a soluble mitochondrial cytochrome c_{550}. Immunoblot experiments clearly indicated a higher steady-state accumulation of this cytochrome at pH 3.7 with respect to pH 6.0. In addition, we showed that the mRNA levels for the cytochrome c_{550} are enhanced at low pH values. It is known that the expression and/or the accumulation of soluble cytochromes *c* in eukaryotes depend on the growth conditions. In *C. reinhardtii*, it has been shown that acetate has a stronger effect on the regulation of the expression of the gene that codes for cytochrome c_{550} than does light intensity. In the presence of acetate, the alga accumulates higher levels of *Cyc* mRNA than in absence of the carbon source [18]. In *S. cerevisiae*, levels of mitochondrial cytochrome c_{550} isoforms are affected by oxygen tension; at increasing oxygen concentrations, the expression of the gene *Cyc7* is switched off while the expression of *Cyc1* is turned on [30]. To our knowledge, this is the first time that the extracellular pH has been shown to influence the level of accumulation of cytochrome *c*. The molecular mechanisms used by *Polytomella* spp. to modulate the expression an accumulation of cytochrome c_{550} in response to extracellular pH remain to be characterized.

Cells grown at pH 3.7 exhibit an enhanced mitochondrial respiration

From our spectroscopic data, it could be inferred that the import of apo-cytochrome *c* into the mitochondria and the heme attachment within the organelle takes place properly in cells grown at pH 3.7. The A_{566}/c_{551} ratio measured for isolated mitochondria was comparable to that for the total cells, indicating that the pool of mitochondrial cytochrome c_{550} was located in mitochondria of cells grown at pH 3.7. The fact that the cytochrome *c* could be reduced by succinate (in the presence of antimycin A and KCN) indicates that this cytochrome participates in the classical respiratory pathway.

The fact that the mitochondrial cytochrome c_{550} was the only cytochrome of the classical respiratory pathway that was overaccumulated at pH 3.7 is puzzling. Our data on oxygen consumption indicate that mitochondria from cells grown at pH 3.7 exhibit higher respiratory rates (20–25%) than mitochondria from cells grown at pH 6.0. The enhanced electron transport activities correlated with an enhanced presence of cytochrome c_{550} in the mitochondria. These data indicate that mitochondrial respiratory capacity in *Polytomella* spp. can be modulated by the concentration of cytochrome c_{550}.

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ARTÍCULO III

The typically mitochondrial DNA-encoded ATP6 subunit of the F_1F_o ATPase is encoded by a nuclear gene in *Chlamydomonas reinhardtii*

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The Typically Mitochondrial DNA-encoded ATP6 Subunit of the F_1F_0-ATPase Is Encoded by a Nuclear Gene in *Chlamydomonas reinhardtii**

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The *atp6* gene, encoding the ATP6 subunit of F_1F_0-ATP synthase, has thus far been found only as an mtDNA-encoded gene. However, *atp6* is absent from mtDNAs of some species, including that of *Chlamydomonas reinhardtii*. Analysis of *C. reinhardtii* expressed sequence tags revealed three overlapping sequences that encoded a protein with similarity to ATP6 proteins. PCR and 5′- and 3′-RACE were used to obtain the complete cDNA and genomic sequences of *C. reinhardtii atp6*. The *atp6* gene exhibited characteristics of a nucleus-encoded gene: Southern hybridization signals consistent with nuclear localization, the presence of introns, and a codon usage and a polyadenylation signal typical of nuclear genes. The corresponding ATP6 protein was confirmed as a subunit of the mitochondrial F_1F_0-ATP synthase from *C. reinhardtii* by N-terminal sequencing. The predicted ATP6 polypeptide has a 107-amino acid cleavable mitochondrial targeting sequence. The mean hydrophobicity of the protein is decreased in these transmembrane regions that are predicted not to participate directly in proton translocation or in intersubunit contacts with the multimeric ring of *c* subunits. This is the first example of a mitochondrial protein with more than two transmembrane stretches directly involved in proton translocation, that is nucleus-encoded.

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*The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AF411119 (*atp6* cDNA sequence from *C. reinhardtii*) and AF411921 (*atp6* genomic sequence from *C. reinhardtii*).*

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The F_1F_0-ATP synthase/ATPase (EC 3.6.1.3) is present in the three domains of life: archea, prokarya, and eukarya. This membrane-bound complex catalyzes ATP synthesis using the electrochemical gradient generated by light-driven or redox-driven electron transfer chains (1). Two main structural domains constitute this oligomeric protein, the membrane-bound sector F_0 involved in proton translocation, and the extrinsic domain F_1 that catalyzes the synthesis of ATP. The F_1 domain contains five subunits in a $3\alpha/3\beta/1\gamma/1\delta/1\epsilon$ stoichiometry (2). The structure of the F_1 sector of the bovine enzyme has been determined crystallographically (3). The F_0 portion of the ATP synthase is less well characterized, due to its highly hydrophobic nature. The structure of a subcomplex of the *Saccharomyces cerevisiae* ATP synthase shows that 10 *c* subunits are arranged around a central stalk (4). In addition, one *a* subunit, also known as ATP6, is thought to interact with the multimeric ring of *c* subunits, translocating protons from the intermembrane space to the mitochondrial matrix. Subunit *a* is predicted to contain two hemichannels that are an obligate route for protons during ATP synthesis driven by the chemiosmotic gradient (5, 6).

In eukaryotic organisms, all of the subunits of the F_1 and a subset of the F_0 portion of the ATP synthase are nucleus-encoded and cytoplasmically synthesized. In most organisms, only two or three of the hydrophobic components of the F_0 sector are mtDNA[1]-encoded. The genes of the F_0 sector that are usually found in the mitochondrial genome are *atp6*, *atp8*, and *atp9* (encoding ATP6 or subunit *a*, ATP8 or A6L, and ATP9 or subunit *c*, respectively). These genes encode highly hydrophobic polypeptides with multiple, putative transmembrane regions (five for ATP6, two for ATP8, and two for ATP9).

The 15.8-kb linear mitochondrial genome of the green alga *C. reinhardtii* lacks several genes that are usually present in mitochondrial genomes, including *nad3*, *nad4L*, *cox2*, *cox3*, *atp6*, *atp8*, and *atp9* (7, 8). These seven genes encode essential components of oxidative phosphorylation complexes and are also absent from the mitochondrial genomes of the related green algae *Chlamydomonas eugametos* (9) and *Chlorogonium elongatum* (10). The genes *cox2a*, *cox2b*, and *cox3*, which encode subunits IIA, IIB, and III of cytochrome *c* oxidase, have been transferred to the nucleus in at least two members of the family

[1] The abbreviations used are: mtDNA, mitochondrial DNA; Cr-ATP6, *C. reinhardtii* subunit ATP6 of mitochondrial F_1F_0-ATP synthase; EST, expressed sequence tag; <H>, local hydrophobicity; MTS, mitochondrial targeting sequence; nt, nucleotide(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Chlamydomonadaceae (11, 12). This gene transfer from the mitochondria to the nucleus was accompanied by several structural changes in these genes. The proteins contain mitochondrial targeting sequences (MTS) and diminished overall mesohydrophobicity that allow the import and assembly of these proteins in the inner mitochondrial membrane.

In this work, the cDNA of the *atp6* gene from *C. reinhardtii* was cloned and sequenced, and the corresponding genomic sequence was obtained. We show that *atp6* is localized in the nuclear genome. The ATP6 polypeptide encoded by this *atp6*, named Cr-ATP6, is homologous to all known ATP6 proteins and is shown to be a constituent of the mitochondrial ATP synthase of *C. reinhardtii*. This is the first description of an *atp6* gene that resides in the nuclear genome and the first example of a nuclear gene that encodes a mitochondrial protein that exhibits structural features characteristic of a proton channel. We demonstrate that the largest decrease in hydrophobicity of Cr-ATP6 occurs in those transmembrane regions that do not participate directly in proton translocation and that are not predicted to interact with adjacent subunits. This characterization of a nuclear version of a gene that is normally present in mitochondrial genomes will facilitate the allotopic expression of mtDNA-encoded genes and its future application to human mitochondrial gene therapy.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—Cell wall-less *C. reinhardtii* strain CW15 was grown in TAP medium (13) with 1% sorbitol (Sigma) under continuous light, with agitation at 100 rpm. The cells were harvested at the late exponential phase of growth.

Nucleic Acid Preparation and Sequencing—Total DNA from *C. reinhardtii* was obtained as previously described (11) or, alternatively, using the DNeasy Plant Mini Kit (Qiagen). Total RNA from *C. reinhardtii* was obtained using the RNeasy Mini Kit (Qiagen). All standard molecular biology techniques were as described (14). Sequencing was performed by the Kimmel Cancer Center DNA Sequencing Facility at Thomas Jefferson University.

Cloning of the cDNA of the Gene atp6 from C. reinhardtii—We identified an EST clone (AV394701) (15) as a fragment of the *atp6* gene from *C. reinhardtii*, because the deduced amino acid sequence at the 3′ end of the EST exhibited high similarity with other ATP6 subunits. The 5′ end showed sequence similarity to two additional clones (AV388269 and AV395475) (15). Based on these EST sequences, two deoxyoligonucleotides were designed (5′-GAGGGTCTTCGGCCTCTTGG-3′ and 5′-CGAAGAACGACAGCGAGAAAGG-3′) and used to amplify a PCR product of 822 nt, containing a portion of the *atp6* cDNA, using a *C. reinhardtii* cDNA library (16) as template. The sequences at the 5′ and 3′ ends of the cDNA were obtained following RACE PCR (17). The primers used were as follows: 5′ RACE, forward (oligo(dT)/adapter) and reverse (5′-CGCCAGAAGCGGTAGATGCC-3′); for nested PCR, forward (oligo adapter) and reverse (5′-GCAGCGAATGGCACCATCG-3′); 3′ RACE, forward (5′-CTACGTTGGCGAGTTCAACAAGC-3′) and reverse (oligo(dT)/adapter); for nested PCR, forward (5′-GTGGTCAA-GAAGGCGCTGTAAGC-3′) and reverse (oligo adapter). The sequences of the dT/adapter and adapter deoxyoligonucleotides were described previously (11). The first cDNA strand for these reactions was obtained using the kit Omniscript (Qiagen) with a specific primer (5′-GAG AAG CCC AGC TTG TAC AGA CC-3′) to obtain the 5′ end and oligo(dT) to obtain the 3′ end of the cDNA.

Cloning of the atp6 Gene from C. reinhardtii—Three pairs of deoxyoligonucleotides were designed using the cDNA sequence to amplify the genomic sequence for the *atp6* gene (5′-AGACGAAGAAT-ATAGATTGG-3′ and 5′-CGCCAGAAGCGGTAGATCCC-3′; 5′-CATTC-GCTGCCCAGCAGGGC-3′ and 5′-GCGAAGAACGACAGCGAGAAGG-G-3′; 5′-CCTCCAACTTGCTGGGTCTGGTG-3′ and 5′-ACGAAGCTTA-CAGTCTCCTC-3′). The PCRs were performed with the *Pfu* Turbo DNA polymerase (Stratagene). For PCR amplification, samples were denatured for 5 min at 94 °C; subjected to 10 cycles of 10 s of denaturation at 94 °C, 1 min of annealing at 55 °C, and 4 min of extension at 68 °C; and subjected to 25 cycles of 10 s of denaturation at 94 °C, 1 min of 55 °C annealing, and 4 min (plus 10 s each cycle) of 68 °C extension. Three different overlapping PCR products of 959, 1973, and 2030 nt were obtained and cloned into pGEM-T Easy Vector (Promega) after the addition of terminal Adenines.

Mitochondrial Protein Analysis—Mitochondria from *C. reinhardtii* were isolated as described (18). The final mitochondrial pellet was resuspended in 35 ml of Percoll dilution buffer with a protease inhibitor mixture (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 mM ε-aminocaproic acid). Mitochondria were diluted to a final concentration of 5 mg of protein/ml, solubilized in 1% dodecylmaltoside, and subjected to blue native electrophoresis using a gradient of 5–12% acrylamide (19). Respiratory chain complexes were separated in the second dimension by Tricine-SDS-PAGE (12% acrylamide) (20). Apparent molecular masses were calculated using prestained molecular weight markers (BenchMark Prestained Protein Ladder; Invitrogen). Protein concentrations were determined according to Markwell *et al.* (21). The isolation of polypeptides for N-terminal sequencing was done as previously described (22). N-terminal sequencing was carried out by Dr. J. d'Alayer on an Applied Biosystems Sequencer at the Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris.

Sequence Analysis in Silico—ATP6 sequences used in this work for mesohydrophobicity analysis were obtained from the SwissProt and TrEMBL data bases at the European Bioinformatics Institute (available on the World Wide Web at srs.ebi.ac.uk). The accession numbers are as follows: Q31720 (*Brassica napus*); P07925, Q36271 (*Zea mays*); P26853 (*Marchantia polymorpha*); P05500 (*Oenothera bertiana*); P05499 (*Nicotiana tabacum*); Q04654 (*Vicia faba*); P20599 (*Triticum aestivum*); P92547, P93298 (*Arabidopsis thaliana*); O21786 (*Oryza sativa*); Q36513 (*Platymonas subcordiformis*); Q35748 (*Raphanus sativus*); Q35781 (*Sorghum bicolor*); Q36376, Q36379 (*Helianthus annuus*); Q37624 (*Prototheca wickerhamii*); Q36730 (*Petunia parodii*); Q34008, Q34004, Q9XPH3, Q9XPH4, Q9TGM2 (*Beta vulgaris*); O79682 (*Glycine max*); Q9ZY27 (*Pedinomonas minor*); Q9XL97 (*Solanum tuberosum*); Q9TC92 (*Nephroselmis olivacea*); Q9MGK0 (*Scenedesmus obliquus*). Alignments were carried out with the ClustalX program (23) with default parameters. Protein weight matrix was Gonnet, although results obtained with Blosum and Pam were similar. The hydrophilic gap penalty was enabled.

Mitochondrial targeting sequence analysis utilized the program MitoProt II (24). The same program was used to calculate the segments with high local hydrophobicity (<H>) in a distance comprising 13–17 amino acids. The mesohydrophobicity was estimated by scanning each sequence for a maximum average hydrophobicity measured in windows from 60 to 30 amino acids and averaging the values. We used several hydrophobicity scales to reduce the possibility of bias.

Protein transmembrane regions and secondary structure were predicted using the program TopPred II (25), the PredictProtein Web server (www.es.embnet.org/Services/MolBio/PredictProtein/) (26), and the ExPASy Molecular Biology Server (www.expasy.ch).

Data used to analyze the *atp6* gene codon usage were obtained from the Codon Usage Database (www.kazusa.or.jp/codon/) with accession numbers [gbpln]:237 for nuclear genes and [gbpln]:21 for mitochondrial genes of *C. reinhardtii*.

RESULTS

Characterization of the atp6 cDNA from C. reinhardtii—We identified a *C. reinhardtii* EST sequence as a partial fragment of an *atp6* cDNA by the similarity of the predicted translation product with known plant ATP6 proteins. This sequence was used to identify two additional, partially overlapping, EST sequences as potential components of a full-length *atp6* cDNA, although portions of the predicted polypeptide lacked similarity with known ATP6 proteins. Based on these EST sequences, deoxyoligonucleotides were designed and used for PCR amplification. A PCR product of 822 nt was obtained using a *C. reinhardtii* cDNA library as template. The sequence of the amplified product confirmed the co-linearity of the EST sequence fragments. The 5′- and 3′-ends of the cDNA were obtained by RACE PCR (17) using cDNA made from *C. reinhardtii* total RNA. The full-length cDNA was PCR-amplified as two overlapping fragments and sequenced, and a length of 2349 nt was obtained for the *atp6* cDNA.

The *C. reinhardtii atp6* cDNA contains a 5′-untranslated region of 27 nt followed by an open reading frame of 1014 nt (Fig. 1A). The sequence flanking the proposed open reading frame initiating methionine codon, AACCATGG, is a consensus translation initiation site (A/C)A(A/C)(A/C)ATG(G/C) for *C. reinhardtii* (27). The TAA stop codon corresponds to the one

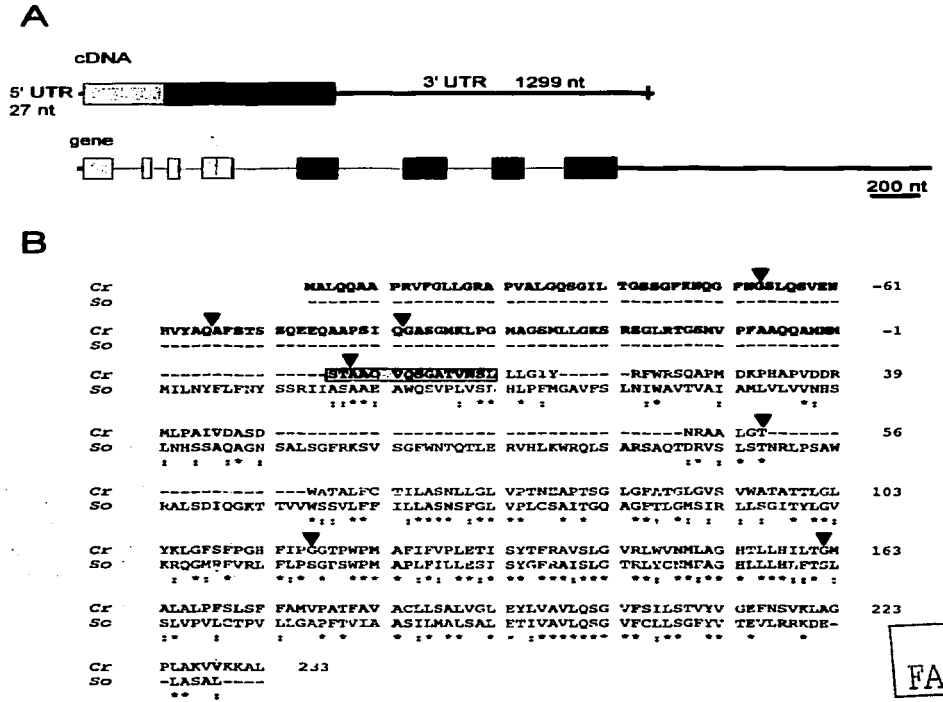

FIG. 1. **Organization of the *atp6* gene of *C. reinhardtii* and sequence comparison of *C. reinhardtii* and *S. obliquus* ATP6 subunits.** *A*, diagram of the organization of the *C. reinhardtii atp6* gene. The coding regions of the *atp6* cDNA and *atp6* gene are shown as *boxes*, the nontranslated regions are shown as *thick black lines*, and introns are indicated by a *thin black line*. The putative mitochondrial targeting sequence is *gray* and the polyadenylation signal is indicated as a *vertical black bar*. *B*, the amino acid sequence alignment of the ATP6 proteins of the F_1F_o-ATP synthase from *C. reinhardtii* (*Cr*) and *S. obliquus* (*So*) (28) are shown. *Black triangles* indicate the positions of introns in the corresponding gene sequence of *C. reinhardtii*. The *C. reinhardtii* sequence is *numbered*, using 1 as the first amino acid of the mature protein. The amino acids of the MTS have negative numbers. The MTS of the *C. reinhardtii* protein is in *boldface type*. The amino-terminal sequence of mature Cr-ATP6 obtained by Edman degradation is *boxed*. *, identical amino acids: :, similar amino acids. The sequence of the mature Cr-ATP6 is 39% identical and 46% similar to that of the *S. obliquus* ATP6. *UTR*, untranslated region.

present in the majority of nuclear genes (70% of the reported genes use this stop codon), and the flanking nucleotides GTAAG are identical to the consensus sequence (G/C)TAA(G/A), characteristic of *C. reinhardtii* nuclear genes (27). The deduced sequence predicts a Cr-ATP6 preprotein of 340 amino acids. A BLAST search of the SwissProt nonredundant protein data base using the Cr-ATP6 sequence produced the highest similarity with the mtDNA-encoded ATP6 subunit from the green alga *S. obliquus* (28, 29), also a chlorophyte alga. The two proteins shared 39% identity and 46% similarity (Fig. 1*B*). The similarity is highest at the carboxyl-terminal region of the protein (59% similarity over amino acids 114–233).

The *atp6* cDNA contains a 3'-untranslated region of 1299 nt (Fig. 1*A*). Two different putative polyadenylation signals were found in the Cr-ATP6 cDNA: TGTAA, the typical signal of nuclear genes of this alga, located 15 nt before the end of the cDNA sequence, and TGTAG, a variation of the most common signal (27), located 1048 nt upstream from the first polyadenylation site. When total RNA was isolated and hybridized with the *atp6* probe in a Northern blot analysis, a single band of 2.4 kb was observed. This result suggested that only the orthodox TGTAA polyadenylation site is functional in the *atp6* RNA sequence (Fig. 2*C*).

Genomic Sequence of atp6 from C. reinhardtii—The complete genomic sequence of the *atp6* gene was obtained following PCR amplification of three overlapping genomic regions using prim-

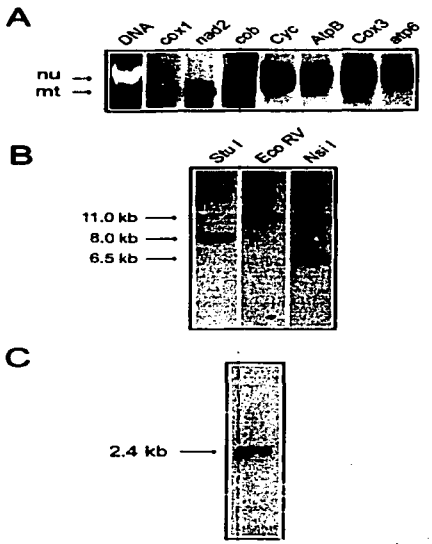

FIG. 2. **The *atp6* gene is nucleus-localized, present in a single copy, and expressed in *C. reinhardtii*.** *A*, nuclear localization of the *atp6* gene. Thirty micrograms of total DNA from *C. reinhardtii* was electrophoresed through a 0.7% agarose gel. The *first lane* (*DNA*) shows the ethidium bromide-stained gel. The *subsequent lanes* show Southern blot analyses of the same gel hybridized with different mtDNA-encoded (*cox1*, *nad2*, and *cob*) and nucleus-encoded (*Cyc*, *AtpB*, and *Cox3*) gene probes. The *atp6* probe hybridized with the nuclear DNA and not with the mtDNA. *B*, *atp6* is a single copy gene. Total DNA from *C. reinhardtii* was digested with the restriction enzymes *Stu*I, *Eco*RV, and *Nsi*I and subjected to Southern blot analysis with a labeled probe for the *atp6* gene. Single hybridizing fragments were detected, suggesting the presence of a single *atp6* gene. *C*, Northern blot analysis of total RNA from *C. reinhardtii*. Twenty micrograms of total RNA from *C. reinhardtii* was electrophoresed through 1% agarose, 0.66 M formaldehyde gels and subjected to Northern blot analysis with a labeled probe for the *atp6* gene. A single hybridizing band of 2.4 kb was detected.

ers derived from the cDNA sequence. The 3577-nt gene contained seven introns, ranging from 72 to 263 nt in length. These introns were present in the coding regions for both the mitochondrial targeting sequence and the mature portion of the protein (Fig. 1, *A* and *B*).

The atp6 Gene Is Located in the Nucleus in C. reinhardtii—When total DNA isolated from *C. reinhardtii* was electrophoresed on agarose, the mtDNA separated as a discrete band running below the major band representing nuclear and chloroplastic DNA (11). Southern blot analysis was carried out to ascertain if the *atp6* gene was present in the nuclear genome. The lower band hybridized with three different mtDNA probes from *C. reinhardtii*, *cox1*, *nad2*, and *cob* (7). In contrast, nuclear DNA hybridized with the *atp6* gene, obtained in this study, and three probes derived from *C. reinhardtii* nuclear genes: *Cyc* encoding cytochrome *c* (30), *AtpB* encoding the β subunit of the ATP synthase (16), and *Cox3* encoding subunit

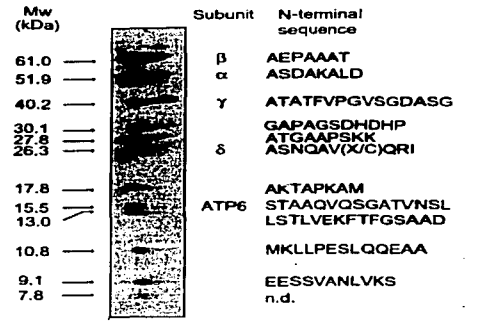

FIG. 3. **Subunit composition of the F_1F_o-ATP synthase complex from *C. reinhardtii*.** The mitochondrial respiratory chain complexes were separated by blue native electrophoresis and in the second dimension by Tricine-SDS-PAGE. Shown is the ATP synthase complex after electrophoresis on a Tricine-SDS-polyacrylamide gel. The amino-terminal sequences of the polypeptides were determined, and the identified subunits are indicated. *n.d.*, not determined.

III of cytochrome *c* oxidase (11) (Fig. 2*A*). A second Southern blot analysis was carried out to determine whether the *atp6* gene was present as a single copy gene in the genome of *C. reinhardtii*. Total DNA was digested with three different restriction enzymes. Southern analysis utilizing an 822-nt PCR product of the coding region of the *atp6* gene as a probe, resolved a single band in each of the restriction digests, suggesting that this gene is present in a single copy in the *C. reinhardtii* genome (Fig. 2*B*).

The pattern of codon utilization of the *atp6* gene was compared with the pattern of codon usage of other known nuclear, chloroplast, and mitochondrial genes of *C. reinhardtii*. Each codon family of the *atp6* gene favored C or G in the third position and is therefore typically nuclear and different from mitochondrial and chloroplast genes (27) (data not shown).

ATP6 Is a Constituent of F_1F_o-ATP Synthase of C. reinhardtii—If the nuclear localized *atp6* gene of *C. reinhardtii* is expressed, the corresponding Cr-ATP6 subunit should be present in the F_1F_o-ATP synthase complex. To identify the Cr-ATP6 subunit, isolated mitochondria from *C. reinhardtii* were solubilized and subjected to blue native electrophoresis followed by second dimension analysis by denaturing Tricine-SDS-PAGE. The F_1F_o-ATP synthase was identified by its characteristic electrophoretic pattern on the second dimension gels. Twelve different polypeptides were present in this complex (Fig. 3). The three major subunits were identified as subunits α, β, and γ, since their apparent molecular masses are similar to those reported previously (31). Their identities were confirmed by N-terminal sequence analysis of their first five amino acids. The additional eight polypeptides were subjected to more extensive N-terminal sequencing (Fig. 3). The 26.3-kDa subunit was identified as subunit δ of F_1F_o-ATP synthase because of its sequence similarity with other δ subunits. The 15.5-kDa polypeptide was identified as subunit ATP6, the protein product encoded by *atp6*, since its initial 15 amino acids matched exactly amino acids 108–122 of the deduced protein sequence encoded by the *atp6* cDNA. This confirms that the *atp6* gene product is a constituent of the F_1F_o-ATPase complex of *C. reinhardtii*. The N-terminal sequences of the eight remaining

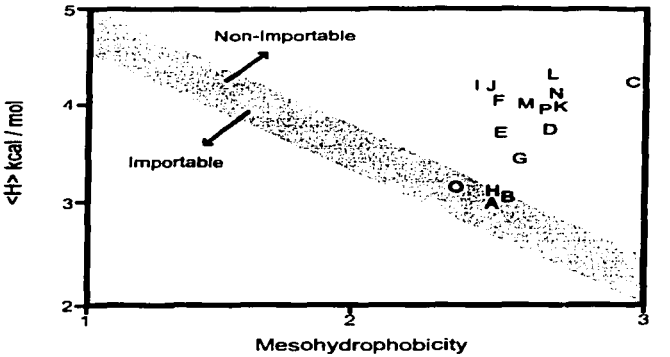

FIG. 4. **Plot of mesohydrophobicity *versus* maximal local hydrophobicity for ATP6 proteins.** The MitoProtII program was used to calculate local hydrophobicity values (<H>) and mesohydrophobicity values for ATP6 proteins, using the PRIFT scale. Proteins are distributed on the *abscissa* according to their mesohydrophobicity value and on the *ordinate* according to the hydrophobicity of the most hydrophobic 17-residue segment. The hypothetical boundary between importable and nonimportable proteins, indicated by a *broad, gray diagonal*, was derived from Claros *et al.* (39) and Perez-Martínez *et al.* (11). The following proteins with their symbols were analyzed: *C. reinhardtii* (*A*), *S. obliquus* (*B*), *P. minor* (*C*), *H. annuus* (*D*), *P. subcordiformis* (*E*), *Z. mays* (*F*), *P. wickerhamii* (*G*), *O. sativa* (*H*), *S. tuberosum* (*I*), *N. tabacum* (*J*), *B. napus* (*K*), *T. aestivum* (*L*), *M. polymorpha* (*M*), *B. vulgaris* (*N*), *G. max* (*O*), *A. thaliana* (*P*).

polypeptides showed no evident similarity with other ATPase subunits, and their identities remain to be established.

Our data indicate that the mature Cr-ATP6 subunit is a protein of 233 amino acids with an expected molecular mass of 24,577 Da and predicts that Cr-ATP6 has an MTS of 107 amino acids, which is cleaved upon import of this polypeptide into mitochondria. For numbering purposes, the mature protein Cr-ATP6 is considered to start at amino acid number 1, and the amino acids that belong to the putative MTS are numbered accordingly (Fig. 1*B*).

Hydrophobicity and Importability of the Nucleus-encoded Subunit ATP6 from C. reinhardtii—Mitochondrial protein import studies suggest that the highest average hydrophobicity over 60–80 amino acids of a polypeptide chain (mesohydrophobicity), along with the maximum hydrophobicity of the putative transmembrane segments, are useful indicators of the likelihood that a protein could be imported into mitochondria (32). We have previously shown that the transfer of genes from the mtDNA to the nucleus in chlamydomonad algae is accompanied by a decrease of these parameters for the COX IIA, COX IIB, and COX III subunits of cytochrome *c* oxidase (11, 12). The physical characteristics of the predicted Cr-ATP6 polypeptide were examined *in silico* and compared with those of ATP6 subunits encoded by other complete plant and algal mitochondrial *atp6* genes. Fig. 4 shows the plot of mesohydrophobicity *versus* maximal local hydrophobicity (<H>) for Cr-ATP6 and for different mtDNA-encoded ATP6 sequences. When compared with several of its mitochondrial counterparts, Cr-ATP6 displays both decreased <H> and mesohydrophobicity. However, other mtDNA encoded ATP6 subunits, like those from *S. obliquus*, *O. sativa*, and *G. max*, also exhibit reduced mesohydrophobicity and <H>.

The reduction of mesohydrophobicity and <H> depends on two factors: the length of the hydrophilic loops between transmembrane domains and the mean hydrophobicity of each transmembrane segment. The alignment of all available ATP6 sequences reveals that the distances between transmembrane domains have not changed (results not shown). Hence, the mean hydrophobicity of each transmembrane segment ought to be reduced in Cr-ATP6. To determine in which regions the hydrophobicity was diminished, hydropathy plots of Cr-ATP6 were compared with those of ATP6 sequences from plants and algae. Analysis of Cr-ATP6 predicts that it contains at least six hydrophobic regions that could be transmembrane regions (data not shown). However, topological considerations argue against the possibility that all hydrophobic segments span the membrane. The most precise positioning of the putative transmembrane helices was obtained by aligning all reported ATP6 sequences and predicting the secondary structure. This analysis suggested a model in which there are five transmembrane segments, named A–E (Fig. 5). This model is in accordance with the topology of the amino and carboxyl termini and the presence of conserved and functionally required amino acids (33–36).

Establishing the boundaries of the five putative transmembrane regions allowed us to quantitate the mean hydrophobicity for each in *C. reinhardtii* and mtDNA-encoded ATP6 subunits from other algae and plants (Fig. 6). While transmembrane helices D and E exhibit similar <H> values when compared with the mean <H> values in helices D and E of plant and algal mitochondrial sequences, significant differences in <H> values were found in the putative transmembrane regions A, B, and C, which are not believed to participate directly in proton translocation and are not thought to interact with the multimeric ring of *c* subunits. This decrease was more than 50% in helices A and C of Cr-ATP6 in comparison with the <H> values of helices A and C of other mtDNA-encoded ATP6 subunits. We conclude that the Cr-ATP6 subunit exhibits diminished overall hydrophobicity as compared with the majority of mtDNA-encoded homologs and that the main decrease in hydrophobicity occurred in those transmembrane regions that exhibit poor sequence conservation and that seem not to be critical for proton translocation.

DISCUSSION

The Gene atp6 of C. reinhardtii Exhibits Nucleus-encoded Characteristics—The 15.8-kb linear mitochondrial genome from *C. reinhardtii* lacks several genes that are typically mtDNA-encoded, including three genes that encode subunits of the F_1F_0-ATP synthase (7). In this work, we show that the *atp6* gene was transferred from the mitochondrial genome to the nucleus in *C. reinhardtii* and demonstrate that the ATP6 protein is present in the mitochondrial F_1F_0-ATP synthase. The *atp6* gene has been previously found only in mitochondrial genomes. This is the first biochemical and genetic evidence for the nuclear localization of *atp6*. We show that the *atp6* gene is

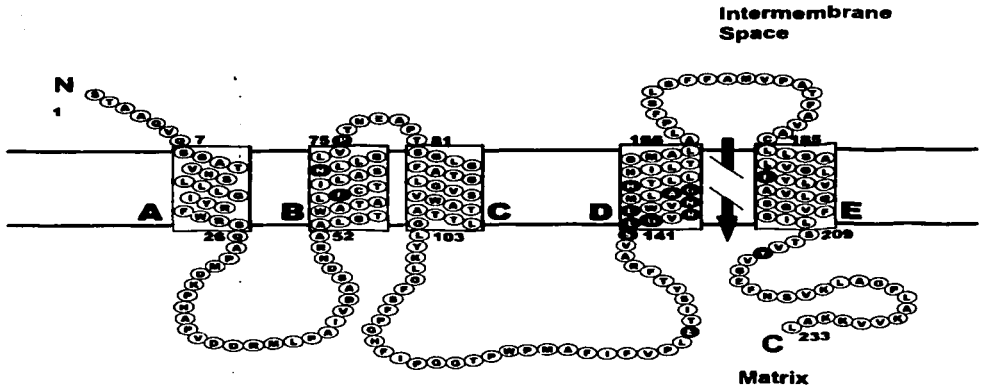

FIG. 5. **Model for the transmembrane helices of the ATP6 subunit of the F_1F_o-ATP synthase from *C. reinhardtii*.** Shown is a model for the location of the transmembrane helices of Cr-ATP6. Conserved amino acids are shown as *black circles*. Transmembrane regions D and E participate in proton translocation. Proton translocation is depicted as a *broken arrow* to illustrate the participation of two hemichannels in subunit ATP6. For simplicity, the multimeric ring of *c* subunits is not depicted.

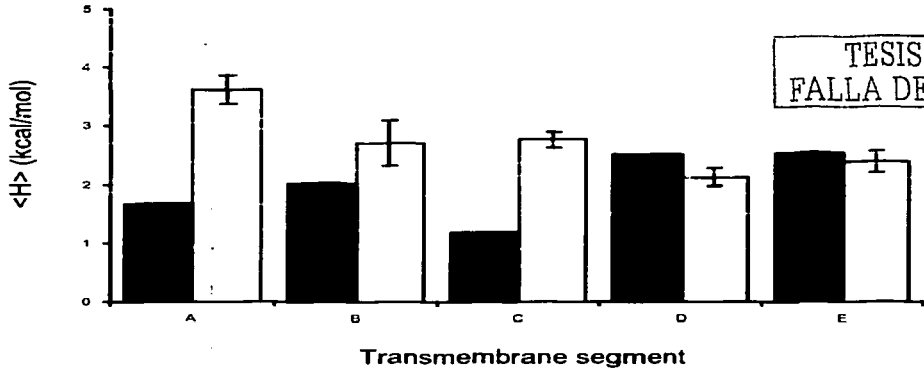

FIG. 6. **Analysis of the mean hydrophobicity values of the five transmembrane regions.** Hydrophobicity values for the five putative transmembrane helices of the Cr-ATP6 (*black bars*) were compared with the mean value of hydrophobicity for 21 mtDNA-encoded ATP6 proteins from plants (*white bars*). In Cr-ATP6, mean hydrophobicity is reduced in transmembrane regions A, B, and C (54, 25, and 57% diminished) as compared with the mean of the mean hydrophobicity values of algal and plant transmembrane regions.

nucleus-localized in *C. reinhardtii* by Southern blot hybridization (Fig. 2*A*). The *atp6* gene exhibits all of the characteristics of a mitochondrial gene that was functionally transferred to the nucleus (37): a nuclear codon usage, a typical nuclear polyadenylation signal, the presence of introns, acquisition of a DNA sequence encoding an amino-terminal MTS, and diminished hydrophobicity relative to many equivalent mtDNA-encoded proteins.

The deduced amino acid sequence of the ATP6 cDNA and the N-terminal sequence of the mature protein predict the existence of a 107-amino acid MTS. This MTS is likely to have been acquired after the original mitochondrial gene was transferred to the nuclear genome. This MTS has a low number of acidic residues, and amino acids 2–24 are predicted to form the amphipathic domain necessary for import of the protein into mitochondria. The methionine (Met^1) adjacent to the MTS cleavage site of the full-length Cr-ATP6 may have been the initiating methionine of the ancestral mitochondrial protein before its corresponding gene was transferred to the nucleus. The MTS is unusually long when compared with other *C.*

reinhardtii MTSs, which exhibit a mean length of 30 amino acids (38). However, we previously found that the nuclear genes encoding COX IIA and COX III of *C. reinhardtii* and *Polytomella* sp., proteins that are typically encoded in the mtDNA, are also predicted to have long MTSs, of 98 and 130 amino acids (11, 12). This suggests that the mitochondrial import of the more hydrophobic mitochondrial proteins, such as COX IIA, COX III, and ATP6, may benefit from an extended MTS.

The duplication of the MTS was found to improve the *in vitro* and *in vivo* import of hydrophobic proteins into yeast mitochondria (32, 39). It was suggested that a long MTS can improve the interaction of the precursor with the mitochondrial import machinery. Alternatively, a long MTS could facilitate the folding of the protein to increase its importability and assembly in the membrane.

The vast majority of the plant mitochondrial ATP6 sequences exhibit physical constraints that are expected to hinder their import into mitochondria (Fig. 4), consistent with their presence in the mitochondrial genome. In contrast, Cr-ATP6 has a lower overall hydrophobicity that allows its import into mitochondria, most probably through the TOM-TIM machinery (40). In addition, some mtDNA-encoded ATP6 sequences also exhibit low <H> and mesohydrophobicity values (*S. obliquus*, *O. sativa*, and *G. max*). These genes seem to be "prepared," from the hydrophobicity point of view, for transfer to the nucleus. We hypothesize that the ancestral Cr-ATP6 protein had a low overall hydrophobicity that enabled the *atp6* gene to be functionally transferred to the nucleus.

Transfer of atp6 and Other mtDNA-encoded ATP Synthase Subunits to the Nucleus—Mitochondrial genomes range widely in size and gene content. Only two genes encoding components of the mitochondrial respiratory chain are invariably present in all mitochondrial genomes known to date: *cob* and *cox1*. Their polytopic protein products are central components of proton-translocating complexes: cytochrome *b* of the bc_1 complex and subunit I of cytochrome *c* oxidase. Cr-ATP6 is the first example of a mitochondrial protein with more than two transmembrane helices, directly involved in proton translocation, that is nucleus-encoded.

The highly reduced 6-kb mtDNA of the parasite *Plasmodium falciparum* contains three genes encoding components of the mitochondrial respiratory chain: *cob*, *cox1*, and *cox3* (41). At the other end of the mtDNA size spectrum, the 60-kb mtDNA of the flagellate *Reclinomonas americana* (42) encodes 23 proteins that participate in oxidative phosphorylation. Five of these are constituents of the F_1F_0-ATP synthase complex: the proteins encoded by the genes *atp1*, *atp3*, *atp6*, *atp8*, and *atp9*. In the mitochondrial genomes of fungi and plants, three genes encoding hydrophobic components of the F_0 sector of F_1F_0-ATP synthase are usually found: *atp6*, *atp8*, and *atp9*. These three genes are absent in the mtDNA of *C. reinhardtii*.

The relocation of *C. reinhardtii atp6* to the nucleus occurred relatively late in evolution, after the massive transfer of genes from the protomitochondrion to the nucleus (43). In the evolution of the green algae, the transfer of *atp6* to the nucleus occurred after the separation of the *Scenedesmus* and *Prototheca* lineages, where *atp6* is still mtDNA-encoded, from the *Chlamydomonas* and *Chlorogonium* lineages, where the *atp6* gene is no longer encoded in the mitochondrial genome.

The transfer of the *atp6* gene to the nuclear genome is unlikely to be exclusive to *C. reinhardtii*. Other organisms that lack the *atp6* gene in their mitochondrial genomes and that are likely to have transferred it to the nucleus are the closely related algae *C. eugametos* (9) and *C. elongatum* (10), the apicomplexan organisms *P. falciparum* (41) and *Plasmodium reinchenowi* (44), and the ciliates *Paramecium aurelia* (45) and *Tetrahymena pyriformis* (46). It would be interesting to examine the sequences of ATP6 from more distantly related organisms to determine whether they have reduced physical constraints for import and to determine where reductions in hydrophobicity occur in the protein.

Mean Hydrophobicity Has Strongly Decreased in Those Transmembrane Regions of Cr-ATP6 That Are Not Critical for Function—The transfer of the *atp6* gene from the mtDNA to the nucleus was accompanied by, or preceded by, a strong decrease in the overall hydrophobicity of the encoded protein (Figs. 4 and 5) and probably facilitates the import and assembly of Cr-ATP6 into an active F_1F_0-ATP synthase. Hydrophobicity analyses, in combination with the alignment of all ATP6 sequences available and previous published observations (33–36), allowed us to predict the presence of five well defined transmembrane regions in Cr-ATP6, named A–E (Fig. 5). The decreased hydrophobicity occurs predominantly in transmembrane regions A, B, and C (Fig. 6), which are poorly conserved among other ATP6 proteins and are thought not to participate directly in the proton translocating function of ATP6. In contrast, the well conserved transmembrane regions D and E maintain similar levels of mean hydrophobicity to mtDNA-encoded ATP6 subunits. We previously observed a similar phenomenon in the COX III proteins of *C. reinhardtii* and *Polytomella* sp. COX III is typically mtDNA-encoded but has been transferred to the nucleus in these algae (11). The nucleus-encoded COX III proteins showed greater diminished hydrophobicity in regions of the protein not in contact with the COX I subunit. This suggests that decreases in mean hydrophobicity of mitochondrial proteins whose genes have been relocated to the nucleus are more likely to occur in regions of the protein not involved in subunit-subunit interactions or in protein function.

Cr-ATP6 contains several amino acids that are conserved in all ATP6 sequences. Helix B ends in a highly conserved Pro and contains two additional conserved amino acids, Phe^{62} and Asn^{69}. The presence of Asn is not very common in transmembrane segments, so we believe its presence may have a role in transmembrane helix association through interhelical hydrogen bonding (47, 48). Transmembrane helices D and E contain most of the conserved amino acids; helix D is preceded by a highly conserved Glu^{131} involved in the proton translocation pathway and contains the invariant amino acids Ser^{141}, Leu^{142}, Gly^{143}, Leu^{146}, Asn^{149}, Ala^{152}, Gly^{153}, and His^{154}. There is also an invariant Arg^{145} that has been previously described as a residue involved in the protonation of the *c* subunit oligomer. Helix E contains the conserved Glu^{194}, which is hypothesized to be involved in proton translocation, and Tyr^{212}, which may be structurally important, after the end of this helix (Fig. 5).

Implications for Human Mitochondrial Gene Therapy—A considerable number of human diseases have been associated with point mutations or deletions in the mitochondrial genome (49–51), and strategies to develop mitochondrial gene therapies have been suggested (52, 53). One promising approach for overcoming mutations in mtDNA-encoded proteins is to place a wild-type copy of the affected gene in the nucleus and target the expressed protein to the mitochondrion to replace the defective mtDNA-encoded protein. Such allotopic expression of a normally mtDNA-encoded gene has been successfully performed in *S. cerevisiae* to overcome a mutation in the mtDNA-encoded *atp8* gene (54).

Analysis of nuclear forms of proteins that are normally encoded in the mtDNA may provide insights that will facilitate the allotopic expression of genes harboring mutations in the mtDNA-encoded genes. The observed decreases in hydrophobicity of nuclear expressed COX III and ATP6 of *C. reinhardtii*

suggest that human mitochondrial genes could potentially be engineered for allotopic expression for gene therapy purposes by modifications to decrease the mean hydrophobicity of the protein product, especially in transmembrane regions that are not highly conserved or that are known to be noncritical for function. Such changes would be in addition to the changes in codon usage and the addition of an MTS-coding sequence that are a prerequisite for nuclear expression of a mitochondrial protein. The presence of long MTSs in COX III, COX IIA, and ATP6 from *C. reinhardtii* suggests that a long MTS would facilitate import in allotopic expression systems. A detailed knowledge of the topology and function of the nucleus-encoded hydrophobic mitochondrial proteins may enhance our understanding of the problems involved in allotopic expression in addition to providing insights into the evolutionary forces that have led to the nuclear localization of genes that are typically mtDNA-encoded.

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ARTÍCULO IV

Structure, organization and expression of the genes encoding mitochondrial cytochrome c_1 and the Rieske iron-sulfur protein in *Chlamydomonas reinhardtii*

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ORIGINAL PAPER

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Structure, organization and expression of the genes encoding mitochondrial cytochrome c_1 and the Rieske iron-sulfur protein in *Chlamydomonas reinhardtii*

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Abstract The sequence and organization of the *Chlamydomonas reinhardtii* genes encoding cytochrome c_1 (*Cyc1*) and the Rieske-type iron-sulfur protein (*Isp*), two key nucleus-encoded subunits of the mitochondrial cytochrome bc_1 complex, are presented. Southern hybridization analysis indicates that both *Cyc1* and *Isp* are present as single-copy genes in *C. reinhardtii*. The *Cyc1* gene spans 6404 bp and contains six introns, ranging from 178 to 1134 bp in size. The *Isp* gene spans 1238 bp and contains four smaller introns, ranging in length from 83 to 167 bp. In both genes, the intron/exon junctions follow the GT/AG rule. Internal conserved sequences were identified in only some of the introns in the *Cyc1* gene. The levels of expression of *Isp* and *Cyc1* genes are comparable in wild-type *C. reinhardtii* cells and in a mutant strain carrying a deletion in the mitochondrial gene for cytochrome *b* (*dum-1*). Nevertheless, no accumulation of the nucleus-encoded cytochrome c_1 or of core proteins I and II was observed in the membranes of the respiratory mutant. These data show that, in the green alga *C. reinhardtii*, the subunits of the cytochrome bc_1 complex fail to assemble properly in the absence of cytochrome *b*.

Keywords Expression of nucleus-encoded genes · Introns · Internal conserved sequences · Mitochondrial targeting sequences · Assembly of protein complexes

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Introduction

Ubiquinol-cytochrome *c* reductase, or the bc_1 complex (EC 1.10.2.2), is a central component of oxidative phosphorylation in eukaryotic and many prokaryotic organisms (Berry et al. 2000). The eukaryotic form of the complex catalyzes the ubiquinol-dependent reduction of cytochrome *c* coupled with proton translocation across the inner mitochondrial membrane. It is made up 10–11 subunits, three of which, cytochrome *b*, cytochrome c_1, and a Rieske-type iron-sulfur protein (ISP), contain redox centers. Apart from cytochrome *b*, all subunits of the bc_1 complex are encoded by nuclear genes, and synthesized in the cytosol as precursors containing mitochondrial targeting sequences (MTS). These precursors are directed to mitochondria, imported, and assembled into the mature respiratory complex (Tzagoloff 1995).

The unicellular photosynthetic alga *Chlamydomonas reinhardtii* has become a model organism for the study of chloroplast biogenesis and flagellum assembly (Harris 2001). Its mitochondrial genome has been sequenced and characterized (Michaelis et al. 1990). However, less attention has been given to the nuclear genes encoding mitochondrial components that participate in oxidative phosphorylation, such as the genes encoding subunits of

the bc_1 complex. The *C. reinhardtii* bc_1 complex is composed of nine subunits (Atteia 1994a). Two genes that encode polypeptides of the *C. reinhardtii* bc_1 complex have been characterized: the mitochondrial cytochrome *b* gene (*cob*) (Michaelis et al. 1990), and a cDNA for the Rieske-type iron-sulfur protein (*Isp*; Atteia and Franzén 1996). We report here the genomic sequence of the *C. reinhardtii* Isp gene and the cDNA and genomic sequences of the *Cyc1* gene for cytochrome c_1. The expression of these genes in wild-type cells and in the respiration-deficient mutant *dum-1* was also investigated.

Materials and methods

Strains and culture conditions

Two *C. reinhardtii* strains were used in this work: the wild-type (137c) and the mitochondrial mutant *dum-1* (Matagne et al. 1989). Both strains may be obtained from the Chlamydomonas Genetics Center (Duke University, Durham, N.C.). The cells were grown in TAP medium (Harris 1989) with agitation under continuous light, at 25°C.

Cloning of the *C. reinhardtii* cDNA that encodes cytochrome c_1

Screening of a *C. reinhardtii* λgt10 cDNA library (Atteia and Franzén 1996) was done by plaque hybridization using an 800-bp fragment of the *Polytomella* sp. cytochrome c_1 gene (Gutiérrez-Cirlos et al.1994) as a probe. The cDNA clones isolated from the positive plaques were excised from λ phage DNA and subcloned in the *Eco*RI site of the plasmid vector pTZ18U (USB).

Screening of *C. reinhardtii* genomic libraries and subcloning

BAC genomic clones containing the genes for *C. reinhardtii Cyc1* (clone address: 37/F05) and *Isp* (clone address: 11/B6) were identified using as probes the respective cDNAs, and obtained from Genome Systems Inc. (St. Louis, Mo.). The strategy used to isolate the *Isp* gene consisted of amplification by PCR of the corresponding genomic sequence using the BAC plasmid as template and two gene-specific primers based on the 5' and 3' ends of the cDNA coding region, IspFW (5'-ATGGCTCTCCGGCGAGCGGTC-3') and IspBW (5'-GCCGATCACCACCTTCTGGCC-3'). For PCR amplification using *Pfu* polymerase (Stratagene), samples were denatured for 5 min at 94°C, and subjected to three cycles of 1 min denaturation at 94°C, 45 s annealing at 55°C, and 3 min extension at 72°C, followed by 27 cycles of 1 min denaturation at 94°C, 45 s annealing at 58°C, and 3 min extension at 72°C. The 1.3-kb PCR product was sequenced and then used as a probe to screen digests of the BAC obtained with different restriction enzymes. A genomic fragment of 2.84 kb resulting from digestion of the BAC with *Bam*HI was cloned in pTZ19R (USB) and sequenced. A diferent strategy was used to obtain the *C. reinhardtii Cyc1* gene, because no PCR product could be obtained from the BAC genomic clones using specific primers. Using the *Cyc1* cDNA as template, the *Cyc1* ORF was first amplified by PCR; the primers used were Cyc1FW (5'-ATGAGGACAAGCCTACTTCGC-3') and Cyc1BW (5'-GTTGACGACGTCCATGACGATGCG-3'). For PCR amplification using *Taq* polymerase (Qiagen), samples were denatured for 5 min at 94°C and subjected to 30 cycles of 1 min denaturation at 94°C, 45 s annealing at 58°C, and 3 min extension at 72°C. The 800-bp amplification product was used as a specific probe to identify fragments of *C. reinhardtii* BACs containing the cytochrome c_1 gene. Two *Pst*I fragments of approximately 5.0 and 2.0 kb that hybridized with the 5' and 3' ends of the *Cyc1* cDNA, respectively, were subcloned in pTZ19R. Comparison of the *Cyc1* cDNA sequence with the genomic sequence allowed the identification of six introns. The region that comprised intron 6 of the *Cyc1* gene proved difficult to sequence, and had to be subcloned independently as a *Pvu*II fragment. In addition, special conditions were required during automatic nucleotide sequencing (see below). The sequences of the two *Pst*I fragments were assembled using a 1.2-kb *Ava*I fragment that also hybridized with the *Cyc1* cDNA probe.

DNA sequencing, sequence analysis and database accession numbers

Sequencing was performed by MWG-Biotech, using an ABI PRISM3700 DNA Analyzer (Applied Biosystems). Sequencing of intron 6 of the *C. reinhardtii* Cyc_1 gene was carried out at the Unidad de Biología Molecular (Instituto de Fisiología Celular, UNAM) using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequencing reactions were performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), with the thermal cycling protocol recommended by the manufacturers, which consisted of an initial denaturation at 96°C for 5 min, followed by 45 cycles of 96°C for 10 s, 50°C for 5 s, an extension step of 4 min at 60°C, and a final rapid thermal ramp to 4°C. Using these conditions, the nucleotide read length of intron 6 was extremely short, and no reliable sequence could be obtained. In order to obtain the full-length sequence of intron 6 on both strands, we used a cycling protocol in which 5% DMSO was added to the reaction and the temperature of the extension step was lowered to 51°C. The sequences obtained in this work are available in the DDBJ/EMBL/GenBank nucleotide sequence database under the Accession Nos. AF245393 (*C. reinhardtii* Cyc_1 cDNA), AJ417788 (*C. reinhardtii* Cyc_1 genomic DNA), and AJ320239 (*C. reinhardtii Isp* genomic DNA).

DNA and RNA isolation, electrophoresis and blotting onto nylon membranes

Total *C. reinhardtii* DNA, isolated according to Newman et al. (1990), was digested with restriction enzymes, fractionated on a 1% agarose gel, and transferred onto Hybond-N^+ membranes (Amersham Pharmacia Biotech) according to standard protocols (Sambrook et al.1989). Membranes were hybridized overnight with homologous probes described below, and further washed under high-stringency conditions (two washes in 0.2×SSC, 0.5% SDS, at 65°C). Total RNA was isolated from wild-type and *dum-1* cells using the RNeasy Mini Kit (Qiagen). Hybridization was carried out overnight as described in Atteia et al. (2000). The membranes were washed in 1×SSC, 0.5% SDS, and 50% formamide at 42°C for 20 min. Southern and Northern blots were hybridized with the indicated DNA probes labeled with [α-^{32}P]dCTP using a Random Primer labeling kit (GibcoBRL).

Protein analysis

Wild-type *C. reinhardtii* and mutant *dum-1* cells were harvested in late exponential phase and total membranes were purified as described by Chua and Bennoun (1975). Chlorophyll levels were determined according to Arnon (1949). Electrophoresis was conducted on linear 12%–18% polyacrylamide gradient gels containing 8 M urea (Piccioni et al.1981). Protein samples in 80 mM Na_2CO_3-80 mM DTT, were solubilized with 2% SDS in the presence of 12% sucrose (Atteia et al.1992). Heme staining with TMBZ (Fluka) was done as described by Thomas et al. (1976). Immunodetection was carried out using the enhanced chemiluminescence method according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech). The antisera used were directed against core I from *Neurospora crassa*, and against cytochrome c_1

and core II from *Saccharomyces cerevisiae*. Apparent molecular masses were estimated using commercially available low-molecular-mass markers (Protein Ladder, Pharmacia).

Sequence analysis *in silico*

Secondary structure prediction of mitochondrial targeting sequences (MTS) was carried out using the program Protean in the Lasergene sequence analysis package (DNASTAR). Sequence alignment was performed with the CLUSTAL W program (Higgins and Sharp, 1988).

Results

Characterization of a *C. reinhardtii* cDNA encoding cytochrome c_1

A cDNA clone for *Cyc1* was isolated by screening a λgt10 library (Atteia and Franzén 1996) using a heterologous probe from *Polytomella* sp. (Gutiérrez-Cirlos et al.1994). The isolated *C. reinhardtii Cyc1* cDNA (DDBJ/EMBL/GenBank AF245393) did not have a polyA tail but did contain a potential TGTAA polyadenylation site. The cDNA contains a 5′-untranslated region of 45 bp followed by an ORF of 945 bp which encodes the full-length apocytochrome c_1 polypeptide. The first ATG in frame with the coding region probably codes for the first methionine.

Analysis of the deduced sequence of cytochrome c_1

The cytochrome c_1 apoprotein of *C. reinhardtii* is synthesized as a precursor of 314 residues with a putative cleavable MTS of 70 amino acids. The mature protein of 244 residues, with a molecular mass of 26,934, exhibits the N-terminal sequence NEAADGLHAPHYPWG previously determined (Atteia 1994b). The deduced sequence of the *C. reinhardtii* cytochrome c_1 was compared with its homologs from other species (Fig. 1). The *C. reinhardtii* sequence is 56–59% identical to c_1-type cytochromes from plant sources, such as *Solanum tuberosum*, and *Arabidopsis thaliana*. The consensus heme-binding region (Q/E)VC(A/S)(A/S)CH is located between residues Q_{108} and H_{113}. In addition, a stretch of 15 uncharged amino acids near the C-terminus (A_{281} to S_{295}) most probably forms a single transmembrane domain, and anchors the protein to the membrane and to cytochrome *b*, as observed in the three-dimensional structures of bc_1 complexes crystallized from other sources (Berry et al.2000).

Genomic organization of the *C. reinhardtii* cytochrome c_1 gene

A restriction map of the cloned DNA fragments that contain the *Cyc1* gene is shown in Fig. 2. To determine the copy number of the genes encoding cytochrome c_1, genomic DNA was digested with several restriction enzymes, fractionated on an agarose gel, blotted onto nylon membranes and hybridized with labeled cDNA corresponding to the *Cyc1* gene ORF. Unique hybridization bands obtained after treatment of total *C. reinhardtii* DNA with different restriction enzymes suggest that cytochrome c_1 is encoded by a single-copy gene (data not shown).

Genomic DNA encoding the ORF of the *C. reinhardtii* cytochrome c_1 gene, as well as the 5′ and 3′ UTRs, was sequenced on both strands. The first and last codons of the gene are separated by 4736 bp, which is more than five times the length of the corresponding cDNA. Thus, the size of the non-coding regions of the gene considerably exceeds that of the exons. The gene is interrupted by six relatively large introns, whose positions were assigned by comparison of the genomic sequence with the cDNA. All introns showed exon/intron splice junctions that followed the GT/AG rule (Breathnach and Chambon 1981). The sequence of intron 6 was difficult to obtain, and required independent subcloning and special sequencing conditions, as described in Materials and methods. The difficulties are attributable to the presence of multiple copies of the nucleotide pentamers AAGGG, AGGGG, and AGAGG.

Genomic organization of the *C. reinhardtii* gene for the Rieske-type iron-sulfur protein

We previously reported the isolation and sequencing of a cDNA encoding the *C. reinhardtii* Rieske-type iron-sulfur protein (Atteia and Franzén 1996). Based on this sequence, a genomic BAC clone was isolated, and a genomic *Bam*HI fragment of 2.84 kb was subcloned in pTZ19R. The restriction map of the cloned DNA fragment that contains the *Isp* gene is shown in Fig. 2. This sequenced fragment exhibited UTRs of 1013 nt at the 5′ end and of 596 nt at the 3′ end. Comparison of the gene sequence with the cDNA sequence indicated the presence of four introns, ranging from 83 to 167 bp in length. In contrast to the introns in the *Cyc1* gene, the introns in the *Isp* gene are relatively small, and were easily sequenced. Southern analysis of *C. reinhardtii* DNA using the *Isp* ORF as a probe identified fragments of the sizes predicted *in silico* from the genomic DNA sequence, and therefore supports the presence of a single *Isp* gene in *C. reinhardtii* (data not shown).

Expression of the *Cyc1* and *Isp* genes in wild-type and *dum-1* mutant strains

Expression of the *Cyc1* and *Isp* genes was analyzed in two strains of *C. reinhardtii*, the wild-type and the respiration-deficient mutant *dum-1*. The *dum-1* mutant strain has a 1.5-kb deletion in the mitochondrial cytochrome *b* gene (*cob*), and is therefore unable to carry

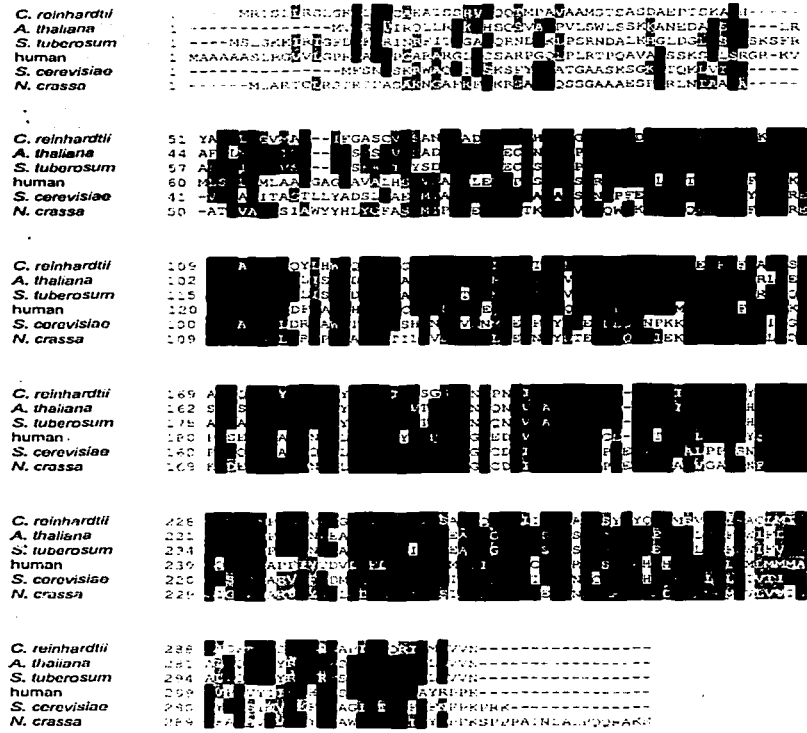

Fig. 1. Multiple protein sequence alignment of c_1-type cytochromes. The *C. reinhardtii* c_1 cytochrome sequence is compared with c_1 cytochromes from *Arabidopsis thaliana* (Kaneko et al.1998), *Solanum tuberosum* (Braun et al.1992), human (Nishikimi et al.1988), *Saccharomyces cerevisiae* (Sadler et al.1984); and *Neurospora crassa* (Römisch et al.1987). Amino acid residues that are conserved in at least 50% of the sequences are shown on a *black* background, similar residues are shown on a *gray* background

out oxidative phosphorylation (Matagne et al.1989; Randolph-Anderson et al.1993). RNA blots loaded with total RNA isolated from the wild-type and mutant strains were first hybridized with a probe containing the *cob* gene (Michaelis et al.1990). As expected, a band of 1.3 kb, corresponding to the cytochrome *b* RNA, was detected in the wild-type strain, but not in the *dum-1* mutant. In contrast, the levels of the *Cyc1* and *Isp* transcripts were comparable in both strains (data not shown). Similar accumulation levels were also obtained with a probe derived from the *Polytomella agilis* β-tubulin B1 gene (*Tub B1*) (Conner et al.1989), which was used as an internal control to check that equivalent amounts of RNA had been loaded in all lanes. These results suggested that the nuclear genes *Cyc1* and *Isp* are expressed in *C. reinhardtii* independently of the mitochondrial *cob* gene.

Accumulation of cytochrome bc_1 subunits

To investigate whether the bc_1 complex subunits were present in the *dum-1* strain, total membranes were isolated from wild-type *C. reinhardtii* cells and the respiration-deficient mutant cells, and their protein contents were analyzed by electrophoresis on a urea/SDS-PA gel. Figure 3 shows polyacrylamide gels stained for proteins with Coomassie-Brilliant (panel A) and for hemes using TMBZ (Thomas et al.1976) (panel B). The TMBZ-stained bands in wild-type *C. reinhardtii* membranes

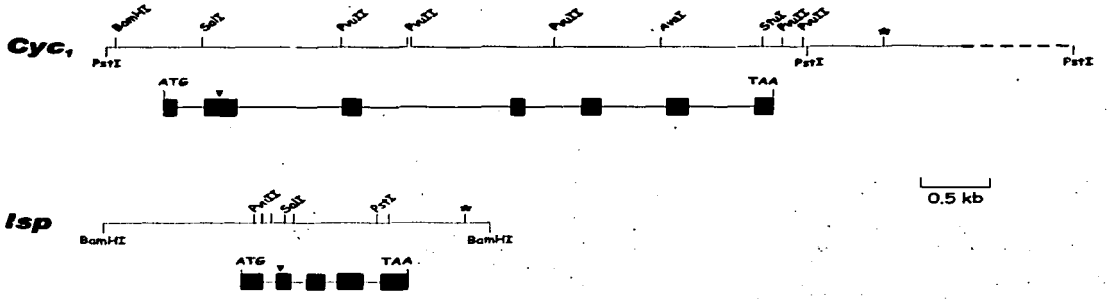

Fig. 2 Physical map and structural organization of the *C. reinhardtii* *Cyc1* and *Isp* genes. The *black boxes* indicate coding regions, *lines* represent introns and 5′ and 3′ flanking regions. For the *Cyc1* gene, only the 5′ end of the 2-kb *Pst*I fragment has been determined; the *dotted line* indicates the region that has not been sequenced. The putative start codon (ATG) and the in-frame stop codon (TAA) are shown in their respective positions. The *vertical arrows* indicate the position of the codon for the first amino acid of the mature protein; *asterisks* show the positions of the putative TGTAA polyadenylation sites. The positions of some of the restriction sites used for Southern analysis are indicated. For the *Isp* gene, the *unlabeled vertical lines* indicate the position of the *Ava*I restriction sites

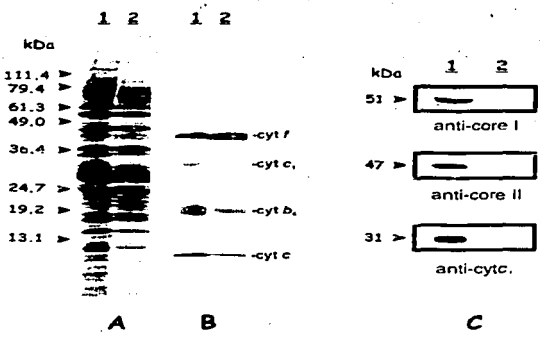

Fig. 3A–C Accumulation of cytochrome bc_1 subunits in total membranes isolated from wild-type *C. reinhardtii* cells and *dum-1*, a mutant strain with a deletion in the mitochondrial *cob* gene. Total membrane polypeptides from wild-type *C. reinhardtii* (1) and *dum-1* (2) strains were fractionated on a 12–18% polyacrylamide gel containing 8 M urea. Membrane samples contained 20 μg chlorophyll each. **A** Coomassie brilliant blue stained gel. **B** TMBZ-stained gel. **C** Immunoblots labeled with antibodies against several subunits of the cytochrome bc_1 complex: *N. crassa* anti-core I protein antibody, and *S. cerevisiae* anti-core II protein and anti-cytochrome c_1 antibodies. The *numbers* indicate apparent molecular masses in kDa

were identified (from top to bottom in Fig. 3B) as chloroplast cytochrome *f*, mitochondrial cytochrome c_1, chloroplast cytochrome b_6, and soluble mitochondrial cytochrome *c*. As shown in Fig. 3B, the TMBZ band that corresponds to mitochondrial cytochrome c_1 is not detectable in the membranes isolated from the mutant strain *dum-1*. The absence of this subunit in the mutant strain was confirmed by immunoblot analysis using an antibody against *S. cerevisiae* cytochrome c_1 (Fig 3C). Figure 3C also shows that antibodies raised against the core proteins I and II recognized the corresponding subunits in membranes from wild-type *C. reinhardtii* cells, but not in membranes isolated from the respiratory mutant *dum-1*. Taken together, these data indicated that cytochrome c_1 and other subunits of the bc_1 complex do not accumulate in cells in the absence of mitochondrial cytochrome *b*.

Discussion

Genomic organization of the *C. reinhardtii Cyc1* gene

We report here the complete cDNA and genomic sequences of the single-copy *Cyc1* gene from *C. reinhardtii*, the first cytochrome c_1 gene sequenced from a chlorophyte alga. The *C. reinhardtii* *Cyc1* gene exhibits all the characteristics of a mitochondrial gene that was functionally transferred to the nucleus early in evolution: typical nuclear codon usage and the presence of polyadenylation signals, DNA sequences encoding N-terminal MTS, and the presence of a relative large number of introns. *Cyc1* genes are also found as single-copy sequences in yeast (Sadler et al.1984) and human (Suzuki et al. 1989). In contrast, two *Cyc1* genes are present in potato plants, and are expressed differentially in various tissues (Braun et al. 1992).

The introns in the *C. reinhardtii* *Cyc1* gene show conserved consensus sequences that surround the splicing sites (Silflow 1998). The introns in the *C. reinhardtii*

Cyc1 gene define seven exons that seem to encode distinct functional domains of cytochrome c_1, a feature previously observed in the corresponding human gene (Suzuki et al. 1989). The MTS of the *C. reinhardtii* cytochrome c_1 is encoded by exon 1 and the first 33 nucleotides of exon 2. These exons seem to encode two distinct domains of the MTS, exon 1 the "mitochondrial targeting signal", and exon 2 the "intramitochondrial sorting signal". However, the region of the gene coding for the MTS is not separated from the region encoding the mature protein by an intron, a feature found in the cytochrome c_1 genes from human (Suzuki et al.1989) and potato (Wegener and Schmitz, 1993). Some additional domains can be identified in the predicted cytochrome c_1 structure: a heme-binding domain (residues Q_{108} to H_{114}) encoded by exon 3; two highly acidic domains that contribute to the interaction with soluble cytochrome *c* (residues E_{140} to E_{152} encoded by exons 3 and 4, and residues D_{239} to D_{246} encoded by exon 6); and the membrane anchor domain necessary for the functional assembly of the protein (residues A_{281} to S_{295}) encoded by the last exon.

Various internal conserved sequences (ICS) have been described for introns of nuclear genes in *C. reinhardtii* (Liss et al. 1997; Funke et al. 1999). These ICS are present in introns larger than 200 bp, and are located either near the 5′ splicing junctions (ICS 1), or near the 3′ splicing junctions (ICS 2) (Pérez-Martínez et al. 2002). Only one ICS 2 (5′-ATCATGAATGTAACCCC-3′) was identified in the *Cyc1* gene of *C. reinhardtii*, near the 3′ splicing junction (nucleotides 420 to 436) of intron 5, which itself is 510 bp long. In addition, a region of intron 3 of the *Cyc1* gene exhibited similarity with an intron in the *C. reinhardtii* gene for the herbicide-resistant protoporphyrinogen oxidase precursor, *Ppx1* (Randolph-Anderson et al. 1998), indicating that the sequence 5′-TTTCCAACCATCCTTGCAACC-3′ may be a previously unrecognized ICS.

Genomic organization of the *Isp* gene of *C. reinhardtii*

The *C. reinhardtii Isp* gene contains four introns, all shorter than the average intron size (219-bp) found in this green alga (Silflow 1998). The MTS is encoded by the first exon; however, it is interrupted by intron 1 shortly before its cleavage site. The other three introns are located in the region that encodes the mature protein. No ICS could be identified in any of these four introns. It is striking that two *C. reinhardtii* nuclear genes encoding redox subunits of the same respiratory complex exhibit such marked differences in the length and complexity of their introns.

Comparison of the *Isp* gene sequence with the *Isp* cDNA sequence published earlier (Atteia and Franzén 1996) revealed some sequence differences in the 5′ UTR. The first 83 nucleotides previously described for the *Isp* cDNA were not found in the *Isp* gene sequence. These 83 nucleotides are identical to nucleotides 325 to 408 of the *C. reinhardtii* solo long terminal repeat retrotransposon TOC1 sequence (Day et al.1988). Other differences between the cDNA and genomic sequences lead to changes in the deduced ISP sequence (P_{45} for Q_{45}, E_{46} for Q_{46}, A_{50} for G_{50}, A_{95} for G_{95}, and R_{96} for E_{97}) and the inclusion of an additional amino acid, T_{96}. Three of these differences occur in the MTS sequence. However, they do not modify the conclusions previously drawn regarding the structure of the *C. reinhardtii* ISP (Atteia and Franzén 1996).

Biochemical characterization of the *C. reinhardtii* mitochondrial respiratory mutant strain *dum-1*

Several mitochondrial mutations that affect the function of respiratory complexes have been characterized in *C. reinhardtii* (Remacle et al.2001). Of relevance to this work is the respiration-deficient mutant *dum-1*, which fails to grow in the dark due to a terminal 1.5-kb deletion in the *cob* gene (Matagne et al.1989; Randolph-Anderson et al. 1993). We analyzed the consequences of the lack of a functional cytochrome *b* gene on the expression and accumulation of the other subunits of the cytochrome bc_1 complex. The levels of the *Cyc1* and *Isp* transcripts were shown to be unaffected by the absence of the mitochondrial *cob* gene, indicating that the mutation does not influence the transcription of *Cyc1* or *Isp*. Immunoblot experiments showed that the *dum-1* strain is deficient in cytochrome c_1 and in two core proteins of the bc_1 complex (Fig. 3). Therefore, it appears that, in *C. reinhardtii*, the subunits of the bc_1 complex are assembled coordinately, and that regulation of the expression of cytochrome c_1 and the ISP takes place at the post-transcriptional level.

In *C. reinhardtii* chloroplasts, the concerted accumulation of the subunits of photosynthetic complexes is the result of the rapid proteolytic degradation of unassembled subunits. The rate of synthesis of some chloroplast-encoded subunits of photosynthetic complexes, known as CES proteins (Controlled by Epistasy of Synthesis), is also regulated by the availability of other subunits of the same complex (Choquet et al. 2001). This work is the first to report the coordinate assembly of the subunits of a mitochondrial respiratory complex in a photosynthetic alga.

Analysis of the MTS encoded by the *Cyc1* and *Isp* genes

The deduced amino acid sequences of the *C. reinhardtii* cytochrome c_1 and ISP, and the previously determined N-terminal sequences of the mature proteins (Atteia 1994b), predict MTSs of 70 and 54 residues, respectively. *C. reinhardtii* MTSs exhibit large variations in size, although they all contain regions with the potential to form positively charged, amphiphilic alpha helices, a

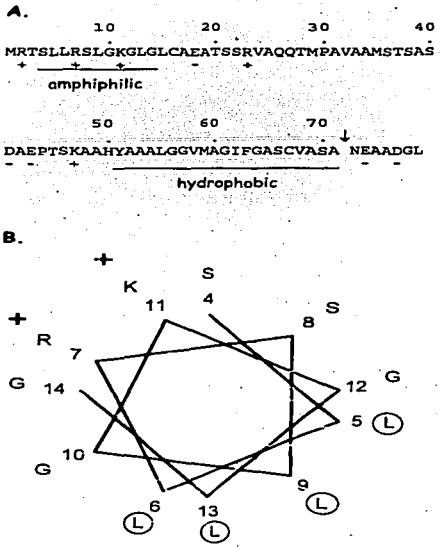

Fig. 4A, B The *C. reinhardtii* cytochrome c_1 MTS. **A** The primary sequence of the MTS. The *arrow* indicates the cleavage site between the MTS and the mature protein. Charged amino acids, a putative amphiphilic alpha helix and a hydrophobic region are indicated. **B** Helical-wheel projection of the *C. reinhardtii* cytochrome c_1 MTS, residues 4–14. Hydrophobic residues are *circled*, and charged residues are indicated by their charges

characteristic feature of MTS (von Heijne et al. 1989). In the cytochrome c_1 MTS, residues 4–14 may form this amphiphilic helix (Fig 4A, B). In the ISP MTS, residues 1–11 form a similar helix (Atteia and Franzén 1996). The MTS of the ISP has also been tested in protein import experiments in vitro (Nurani et al.1997). Incubation of a radiolabeled precursor of the ISP with isolated *C. reinhardtii* mitochondria resulted in the production of the mature form of the ISP. This mature form was protected against externally added protease, indicating that it had been imported into the mitochondria.

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ARTÍCULO V

Bifunctional aldehyde/alcohol dehydrogenase (ADHE) in chlorophyte algal mitochondria

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Bifunctional aldehyde/alcohol dehydrogenase (ADHE) in chlorophyte algal mitochondria

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Running title: aldehyde/alcohol dehydrogenase in unicellular algae

Keywords: chlorophytes, evolution, OXPHOS complexes, pH regulation

SUMMARY

Protein profiles of mitochondria isolated from the heterotrophic chlorophyte *Polytomella* sp. grown on ethanol at pH 6.0 and pH 3.7 were analyzed by Blue Native and denaturing polyacrylamide gel electrophoresis. Steady-state levels of oxidative phosphorylation complexes were influenced by external pH. Levels of an abundant, soluble, mitochondrial protein of 85-kDa and its corresponding mRNA increased at pH 6.0 relative to pH 3.7. N-terminal and internal sequencing of the 85 kDa mitochondrial protein together with the corresponding cDNA identified it as a bifunctional aldehyde/alcohol dehydrogenase (ADHE) with strong similarity to homologues from eubacteria and amitochondriate protists. A mitochondrial targeting sequence of 27 amino acids precedes the N-terminus of the mature mitochondrial protein. A gene encoding an ADHE homologue was also identified in the genome of *Chlamydomonas reinhardtii*, a photosynthetic relative of *Polytomella*. ADHE reveals a complex picture of sequence similarity among homologues. The lack of ADHE from archaebacteria indicates a eubacterial origin for the eukaryotic enzyme. Among eukaryotes, ADHE has hitherto been characteristic of anaerobes. ADHE is essential to cytosolic energy metabolism of amitochondriate protists such as *Giardia intestinalis* and *Entamoeba histolytica*. The abundance and expression pattern suggest an important role for ADHE in mitochondrial metabolism of *Polytomella* under the conditions studied. Presence of ADHE in an oxygen-respiring algal mitochondrion and coexpression at ambient oxygen levels with respiratory chain components is unexpected and inconsistent with the view that eukaryotes acquired ADHE genes specifically as an adaptation to an anaerobic lifestyle.

INTRODUCTION

The colorless chlorophytes of the genus *Polytomella* are members of a single monophyletic clade, the *Reinhardtii* clade (1), and share a common ancestor with their photosynthetic relatives *Chlamydomonas reinhardtii* and *Volvox carterii* (2, 3). *Polytomella* is found in various habitats including fresh water ponds and greenhouse soils (4). In the laboratory the algae can be grown on a great variety of carbon sources and under a wide range of pH (5-8). The growth of this alga is often associated with significant changes in the pH of the culture medium (8). The ability of the alga to adapt to different habitats implies a tight regulation of the intracellular concentration of solutes and protons.

Polytomella sp. is able to grow on ethanol at pH below 7.0 whereby its metabolism tends to acidify the growth medium, although it can also be grown under conditions where the pH is maintained constant (8). Studies on *Polytomella* sp. cells grown on ethanol in the presence of non-metabolizable buffers have shown that the external pH influences the function and biogenesis of mitochondria. The rates of oxygen uptake in the presence of substrates like succinate, malate or ethanol are 20-25% higher in mitochondria isolated from cells grown at pH 3.7 than in mitochondria from cells grown at pH 6.0 (8). The steady-state accumulation of mitochondrial proteins is also affected by the external pH. Mitochondria from cells grown at pH 3.7 contained more polypeptides of 30-kDa or less, one of which was cytochrome *c* (8), relative to mitochondria from cells grown at pH 6.0. At present, the identity of the different mitochondrial protein patterns of cells grown at pH 6.0 and pH 3.7 is not known.

The aim of the present study was to further characterize the influence of the external pH on the mitochondrial protein content in *Polytomella* sp. grown on ethanol and to identify proteins that exhibit a pH-dependent accumulation. Here we report changes in the levels of oxidative phosphorylation (OXPHOS) complexes in mitochondria isolated from *Polytomella* sp. cells grown on ethanol at pH 6.0 and pH 3.7 and in the levels of an 85-kDa soluble protein that we identified, on the basis of its amino acid sequence, as a bifunctional aldehyde/alcohol dehydrogenase (ADHE). A ADHE homologue is also present in *C. reinhardtii*, a photosynthetic relative of *Polytomella*.

EXPERIMENTAL PROCEDURES

Isolation and subfractionation of Polytomella sp. mitochondria

Polytomella sp. (198.80, E.G. Pringsheim) was grown in Erlenmeyer flasks with cotton stoppers allowing for ample gas exchange at room temperature on ethanol at pH 3.7 and at pH 6.0, or on acetate at pH 6.0 (8). Mitochondria were isolated as described (8). Mitochondria, resuspended in 0.2 M mannitol, 5 mM potassium phosphate (pH 7.2) at a concentration of 10-12 mg protein/ml in the presence of 0.5 mM PMSF and 2 mM amino caproic acid, were sonicated three times for 10 sec, and centrifuged for 1 hour at 100,000 x *g*.

Protein Analysis

Mitochondria and mitochondrial subfractions were freshly prepared for BN-PAGE analysis. The soluble mitochondrial fraction was supplemented with 1% dodecyl maltoside (*n*-dodecyl β-D-maltoside) and 0.25% Coomassie Serva Blue G. Mitochondria and mitochondrial membranes were washed twice in 250 mM sorbitol, 15 mM Bis-Tris (pH 7.0); for solubilization, the proteins were resuspended at 15 mM Bis-Tris, 750 mM amino caproic acid (pH 7.0) containing 2% dodecyl maltoside at a final protein concentration of 5 mg/ml. The sample was centrifuged for 20 min at 40,000 x *g;* the solubilized material was then supplemented with Coomassie Serva Blue G (one half of the volume of added dodecyl maltoside). All the samples were loaded on BN-PAGE using acrylamide gradients of 5-12% or 5-15% (9). Staining for NADH dehydrogenase activities on BN-PAGE was performed as in (10). Electroblotting of BN-PAGE

lanes was done as described (11). Immunodetection was carried out by the enhanced chemiluminescence peroxidase method (ECL™, Amersham-Pharmacia Biotech.) using antisera raised against the β-subunit of the bovine mitochondrial ATP synthase, the COXIIA subunit of *Polytomella* sp. cytochrome *c* oxidase, and the core I subunit of *Neurospora crassa* bc_1 complex. Entire lanes of BN-PAGE were used to resolve the proteins in a 2D-Tricine-SDS-PAGE (15% acrylamide) (11). For sequence determination, a lane of BN-PAGE with soluble mitochondrial proteins (1 mg of protein) was resolved on 2D-SDS-PAGE; following electrophoresis, the proteins were electrotransferred onto ProBlot membrane and stained with Coomassie blue R-250 (12). The proteins of interest were excised and subjected to N-terminal or internal sequencing as described (13). Protein concentrations were determined according to Markwell *et al.* (14). Prestained molecular mass markers (Invitrogen) were used.

Isolation of Polytomella sp. ADHE cDNA

Two oligodeoxynucleotides, 5'- GAG CAG AAG TCC AAG TCY GAY GAG G -3' and 5'- CTT CTC RGC RTC RGC GGA RGG -3' were designed from the N-terminal sequence (residues Glu6 to Glu13 of the mature protein) and the internal sequence IS2 (Pro688 to Lys694) of *Polytomella* sp. mature ADHE protein. PCR amplification was carried out using *Taq* DNA polymerase (Qiagen). Total *Polytomella* sp. DNA was denatured for 5 min at 94°C, then subjected to three cycles of 1 min denaturation at 94°C, 45 sec annealing at 60°C, and 3 min extension at 72°C; and subjected to 27 cycles of 1 min denaturation at 94°C, 45 sec annealing at 62°C, and 3 min extension at 72°C. The obtained 2-kb PCR product (p*AdhE*) was cloned into pGEM-T Easy Vector (Promega) and sequenced. p*AdhE* was further used to screen a □ZAPII *Polytomella* cDNA library. The sequence of the longest cDNA isolated (1.6 kb) from 5,000 p.f.u. screened, overlaped the 3'-end of p*AdhE* PCR product by 300 bp. The 5'-end sequence of *Polytomella* sp. ADHE cDNA was determined using the RNA ligase-mediated rapid amplification of cDNAs ends method (RLM-RACE, Ambion), as indicated by the provider, and using total RNA from cells grown on acetate at pH 6.0. The gene-specific primers used were: 5'- GGC TGT AAA CGA ACT CGG AGG CGA AG -3' (corresponding to residues Phe81 to Ser88 of the mature protein) and 5'- GCG GCG CGG AAG ATC TTG TCG -3' (residues Asp42 to Ala47). The PCR steps were carried out at 60°C. Sequencing was done at the Unidad de Biología Molecular (IFC-UNAM) and at MWG Biotech. Inc. (USA).

DNA and RNA analysis

Total *Polytomella* sp. DNA isolated according to Newman *et al.* (15) was digested with restriction enzymes, separated on a 1% agarose gel, and transferred onto Hybond-N^+ membranes (Amersham Pharmacia Biotech.) using standard protocols (16). Membranes were hybridized overnight at 65°C with the p*AdhE* PCR product and washed 2 x 20 min at 65°C in 0.2 x SSC and 0.5% SDS. Total RNA from *Polytomella* sp. cells was isolated using Trizol Reagent (GibcoBRL), separated on a 1% agarose gel, and transferred onto Hybond-N^+ membranes. Hybridization was carried out overnight as previously described (8). The membranes were washed 2 x 20 min, at 42°C in 1 x SSC and 0.5% SDS. DNA probes p*AdhE* (see above) and *TubB1* from *Polytomella agilis* (8, 17) were labeled with [α-^{32}P] dCTP using the Random Primer labeling kit (GibcoBRL).

Sequence Analysis

EST clones of *C. reinhardtii* were obtained from the ChlamyEST database at http://www.biology.duke.edu/chlamy_genome/cgp.html using the WU-TBLASTN program. Search for an ADHE gene in *C. reinhardtii* genome was done using the site http://genome.jgi-psf.org/cgi-bin/browserLoad/3e7f2c99428ddae9031d6856. Protein sequence data were retrieved from Swiss-Prot + TrEMBL (18) and GenBank, non-redundant protein sequence databases (19), using gapped BLASTP program with default gap penalties and BLOSUM 62 substitution matrix (20). Molecular mass and pI were calculated using the Compute pI/MW tool (21). Motif search was done using the Integrated Protein Classification Database (iProClass) (pir.georgetown.edu)

software. Sequences were aligned with ClustalW (22). Protein logdet distances (23) were calculated using the LDDist program available at the website http://artedi.ebc.uu.se/molev/software/LDDist.html and used for constructing neighbor-joining trees (24) and planar networks. Planar networks were constructed with NeighborNet (25) and SplitsTree (26).

RESULTS

Identification of the major OXPHOS complexes from Polytomella sp.

Mitochondria from *Polytomella* sp. cells grown on ethanol at pH 6.0 were solubilized with dodecyl maltoside and analyzed on BN-PAGE. As shown in Fig. 1A, the pattern of *Polytomella* sp. mitochondrial protein complexes contrasts with the well-characterized pattern of beef heart mitochondrial complexes (9). The major *Polytomella* OXPHOS complexes were identified by immunoblot analysis and specific activity staining. An antiserum against subunit □ of bovine complex V (F_0F_1-ATP synthase) detected a single band of at least 1600-kDa on BN-PAGE (Fig. 1B). Thus, like in *C. reinhardtii* (13), *Polytomella* sp. complex V runs as a dimer. The incubation of a BN-PAGE lane with nitro blue tetrazolium and NADH (10) led to the detection of two bands of ~ 980 and 250-kDa exhibiting NADH dehydrogenase activity (Fig. 1B). Based on its mobility on BN-PAGE and on its polypeptide composition (see Fig. 2B), the 980-kDa band was identified as complex I (NADH:Q oxidoreductase); the band of 250-kDa was not identified. The 500-kDa protein complex was assigned to *Polytomella* sp. complex III (QH_2:cyt *c* oxidoreductase), on the basis of its detection with an antiserum against *N. crassa* core I subunit. The position of complex IV (cytochrome *c* oxidase) was determined using an antiserum against the COXIIA subunit (28). As shown in Fig. 1B, this antibody recognized multiple bands on BN-PAGE in the range of 150 to 180 kDa; none of which coincided with the strong band at 200-kDa (Fig. 1B; see below). Therefore, in contrast to complex IV from various sources, including mammals (9), plants (11) and *C. reinhardtii* (13), *Polytomella* complex IV does not appear as a major band on BN-PAGE (Fig. 1A).

External pH affects accumulation of mitochondrial protein complexes

BN-PAGE patterns of mitochondria isolated from *Polytomella* sp. cells grown on ethanol at pH 6.0 and pH 3.7 were qualitatively similar but the relative abundance of protein complexes differed (Fig. 2A). BN-PAGE and 2D-SDS-PAGE (Fig. 2B) showed that the levels of complex V were significantly higher in mitochondria from cells grown at pH 6.0 than in mitochondria from cells grown at pH 3.7.. In contrast, the levels of respiratory complexes I, III, and IV were lower in mitochondria from cells grown at pH 6.0 than in mitochondria from cells grown at pH 3.7.

Besides the proteins of the OXPHOS system, several other proteins showed a pH-dependent accumulation. One of them was a protein of 85 kDa that belongs to the aforementioned 200-kDa complex visible on BN-PAGE above complex IV (Fig 1, 2). Higher contents of the 85 kDa protein were found in mitochondria from cells grown at pH 6.0 (Fig. 2, arrow). The N-terminal sequence of the 85-kDa protein is reported in Table I.

2D-SDS-PAGE analysis of soluble protein complexes in Polytomella sp.

Mitochondria from cells grown on ethanol at pH 6.0 were fractionated into their soluble and membrane-bound components and the protein complexes in the subfractions were further separated on BN-PAGE (Fig. 3, left panel). As expected, the OXPHOS complexes were found in the membrane fraction. In the soluble fraction, two major protein complexes of ~ 200 and 100-kDa were detected (Fig. 3, left panel).

The soluble protein complexes, separated by BN-PAGE, were resolved into their constitutive subunits on a 2D-SDS-PAGE (Fig. 3, right panel). Corresponding to the 200-kDa

range several proteins were resolved, with a major protein of 85-kDa (spot 1) and two additional proteins of 60-kDa (spot 2) and 35-kDa (spot 3). The N-terminal sequence of the protein in spot 1 was identical to the N-terminal sequence of the 85-kDa protein (Table I) indicating that this protein is soluble. The N-terminal sequence of two tryptic fragments obtained from the 85-kDa protein (IS1, IS2; see Table I) did not produce significant hits in database searches. Protein spots 2 and 3 were also subjected to Edman degradation. No N-terminal sequence could be obtained for spot 2, likely because of a blocked N-terminus. Edman degradation of spot 3 gave two amino acids for several cycles (Table I).

Database searching identified several protein spots on 2D gels as typical mitochondrial proteins (Table I). Spot 4 (60-kDa) and spot 5 (70-kDa) were identified as the mitochondrial heat-shock proteins HSP 60 and HSP 70. Spot 6 (45-kDa) and spot 7 (35-kDa) were identified as malate dehydrogenase and citrate synthase (Table I) of the tricarboxylic acid cycle.

Identification of a Polytomella sp. cDNA encoding mitochondrial ADHE

Using primers designed from the peptides obtained from spot 1, its corresponding cDNA was isolated through PCR amplification, cDNA library screening and RLM-RACE. Database searching with the cDNA sequence revealed that spot 1 corresponds to a bifunctional aldehyde/alcohol dehydrogenase, ADHE. *Polytomella* ADHE cDNA encodes a 885-amino acid protein encompassing the N-terminal sequence determined from the mature mitochondrial protein, thus revealing the cleavage site of the 27 amino acid N-terminal mitochondrial targeting sequence (MTS) (Figure A, for reviewers only). The MTS lacks acidic residues, has a high content of basic and hydroxylated residues and, conforms well to MTS prediction programs, including MITOPROT II (29), PREDOTAR (version 0.5, www.inra.fr/predotar/) and TargetP V1 (30). The molecular mass of the mature ADHE was calculated to be 88547-Da and its pI 6.98. Southern hybridization against total *Polytomella* DNA (data not shown) indicates that ADHE is encoded by a single copy gene.

Database searching identified the two distinct enzymatic domains typical of ADHE in the *Polytomella* protein: the N-terminal region (residues Lys20 to Pro455) is homologous to the acetylating aldehyde dehydrogenase (ALDH) family, a member of the ALDH superfamily (aldehyde:NAD^+ oxidoreductases, EC 1.2.1.10), whereas the C-terminal region (Lys485 to Ala854) is homologous to the iron-containing alcohol dehydrogenase family (Fe-ADHE; alcohol:NAD^+ oxidoreductases, EC 1.1.1.1). *Polytomella* ADHE exhibits high similarity (52 to 69%) to ADHE from cyanobacteria, clostridia and enterobacteria; the algal protein also shows similarity (47 to 64%) to ADHE from lactobacilli, bacilli and from the amitochondriate eukaryotes *Giardia intestinalis, Spironucleus barkhanus, Mastigamoeba balmuthii,* and *Entamoeba histolytica* (31, 32).

Polytomella ADHE exhibits several features characteristic of ADHE: the conserved sequences found in CoA-acetylating ALDH, $PxG(x_6)P(x_3)P$ (residues 113 to 126 of the mature protein) (33, 35) and $G(x_6)D(x_7)A(x_7)K(x_4)G(x_2)C$ (residues 224 to 255) (33); the ALDH catalytic center DNGxICASEQ (residues 250 to 259) (31); two nucleotide-binding sites GxGxG (residues 220 to 224) and $GCG(x_2)GG$ (residues 428 to 434) that may be involved in NADH binding (33), and a third nucleotide binding site $GxG(x_2)V(x_3)S$ in the ADH domain (residues 601 to 610) implicated in NAD(P)H binding (33, 34). The C-terminal ADH domain of ADHE shows high similarity with Fe-ADH homologues that use iron to polarize the carbonyl group of acetaldehyde during catalysis. *Polytomella* sp. ADHE also exhibits the two iron-binding motifs conserved in Fe-ADH: the signature 1 (ADH-IRON1; AIVDPSLIAALPKAAVAAGAFEAISHAVE; residues 633 to 661) is highly conserved, while the signature 2 (ADH-IRON2; GVTQSLANKVAVACDIPVGVAAA; residues 711 to 732) lacks three histidine residues conserved in most ADHE homologues (Figure A, for reviewers only). The sequence between the two ADH-IRON signatures is shorter in *Polytomella* sp. than in other ADHE sequences as confirmed by the amino acid sequencing of the tryptic fragment IS2 (Asp674 to Lys694). *Polytomella* ADHE also contains the conserved patterns for ADH type III enzymes $GGG(x_3)D(x_2)K$ (residues 545 to 554) and $A(x_2)DQC(x_2)ANPRxP$

(residues 829 to 842) (33). Finally, unlike bacteria and amitochondriate protists, the linker sequence (31) that connects the ALDH domain with the ADH domain in *Polytomella* ADHE (residues Ala466 to Gly484) is unique in that it lacks charged residues and contains several hydroxylated residues.

C. reinhardtii expressed sequence tags (EST) dataset searching by similarity to known ADHE proteins indicated the presence of an ADHE homologue in the photosynthetic alga. Overlapping EST clones (AV397610, AV639995, AV644998; BQ808648, BI873972, AV624287, BG855351, BQ810550, BI873402 and BG855598), contigs (20021010.5320.2, 20021010.2041.2) and genomic sequence (scaffold 592) allowed to reconstruct *C. reinhardtii* ADHE cDNA sequence. *C. reinhardtii* ADHE is encoded as a 951 amino acids that exhibits an N-terminal extension of approximately 60 residues compared to ADHE in amitochondriate eukaryotes (Figure A, for reviewers only). A mitochondrial localization for *C. reinhardtii* ADHE is predicted with the programs PREDOTAR (version 0.5, www.inra.fr/predotar/) and MITOPROT II (29). *C. reinhardtii* and *Polytomella* sp. ADHE sequences are highly similar (56 % identity), nevertheless differences between the sequences of the putative ADH-IRON 2 signature and between the linker sequences were observed (Figure A, for reviewers only). The complete genomic sequence encoding *C. reinhardtii* ADHE spans 6,358 bp and contains 15 introns.

Expression of Polytomella sp. ADHE

Northern hybridization was performed with RNA isolated from cells grown on ethanol at pH 6.0 or 3.7, and on acetate at pH 6.0. RNA blots were probed with the p*AdhE* PCR product and the *TubB1* probe as an internal control for loading equivalent amounts of RNA. A single *AdhE* transcript of 3.5 kb was detected in all three conditions (Fig. 4). While strong hybridization signals were observed with RNA from cells grown at pH 6.0 on acetate or on ethanol, the signal obtained with RNA from cells grown on ethanol at pH 3.7 was significantly weaker. These data show that *Polytomella* ADHE expression is strongly influenced by the pH of the culture medium.

Phylogenetic analyses of ADHE

Phylogenetic analysis was carried out with ADHE sequences available in databases: four from amitochondriate protists and the remainder from eubacteria. No archaebacterial homologues were detected in database searches. As shown in Fig. 5a, *Polytomella* sp. and *C. reinhardtii* ADHE cluster closely but in a position distinct from *Entamoeba histolytica* ADHE in the bifurcating NJ tree. The ADHE from *E. histolytica* shares higher amino acid sequence identity with homologues from *Pasteurella multocida* (63%) or *Streptococcus pneumoniae* (62%) whereas ADHE from *Polytomella* sp. and *C. reinhardtii* are more similar to the homologue from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (52% and 66% identity, respectively). Notably, the predicted *C. reinhardtii* ADHE shows fewer positional identities to *Polytomella* sp. ADHE (57%) than to ADHE in the cyanobacterium *T. elongatus* (66%), an affinity that is represented as a shared component of similarity by the NeighborNet planar network in Fig. 5b.

DISCUSSION

ADHE in Polytomella sp. mitochondria

ADHE catalyzes the fermentative production of ethanol by two sequential NADH-dependent reductions of acetyl-CoA, releasing ethanol and CoASH (35-37). It is believed that ADHE arose through an ancient gene fusion of an acetyl-CoA-dependent aldehyde dehydrogenase and an iron-dependent alcohol dehydrogenase, probably within the same eubacterial operon (31, 37). In *Salmonella typhimurium eut* operon, the *EutE* gene encoding an acetyl-CoA ALDH is proximal to the *EutG* gene that encodes a Fe-ADH; the EutE and EutG

protein sequences in tandem align with ADHE (38). The ADHE gene occurs in a skew distribution among phylogenetically disparate lineages. The majority of the prokaryotes do not have the gene and among eukaryotes it has only been found in a few amitochondriate protists.

The presence of ADHE in *Polytomella* sp. was shown by amino acid sequencing of the protein identified in isolated mitochondria. The protein is soluble and appears to be mainly present as a homodimer, in contrast to *E. coli* and *E. histolytica*, where ADHE exists as multimers of 20 to 60 protomers (36, 39). Comparison of the N-terminus of mitochondrial ADHE from *Polytomella* to the cDNA sequence reveals that ADHE is encoded as a precursor protein with a typical MTS that is cleaved upon import into the organelle. Based upon sequence similarity and the presence of conserved cofactor-binding signatures, *Polytomella* sp. ADHE is likely to perform the same enzymatic reactions catalyzed by eubacterial ADHE.

The expression of *E. coli* ADHE is anaerobically regulated at both the transcriptional and translation levels (40, 41). During aerobic metabolism, ADHE is highly susceptible to metal-catalyzed oxidation. The amino acid chains in ADHE and, in particular the histidine residues in ADH-IRON 2 signature, are thought to be attacked by highly reactive hydroxyl radicals locally generated by the active site Fe^{2+} of the ADH domain (42, 43). In contrast to *Chlamydomonas*, *Polytomella* ADHE lacks the conserved histidine residues in the ADH-IRON signature 2, suggesting that the protein has lost its iron-binding capacity or that iron chelation involves more distant residues, and also suggesting a lower sensitivity to oxygen.

Site-directed mutagenesis of *E. coli* ADHE showed that the conversion of Glu568 in the ADH domain into virtually any non-acidic residue resulted in an enzyme active under both aerobic and anaerobic conditions; the mutated ADHE allowed *E. coli* to grow aerobically on ethanol (44). The presence of a glycine residue in the algal sequences (Gly569 in *Polytomella* sp. and Gly641 in *C. reinhardtii*) at the equivalent position of the Glu568 in the *E. coli* sequence or of the Ala138 in *S. typhimurium* EutG protein (45) suggests that the algal protein may be able to function as an ethanol dehydrogenase. However, Glu568 in the ADH domain is not an invariable amino acid, for example it is replaced by Ala578 in the anaerobic protist *E. histolytica*.

External pH and the role of ADHE in Polytomella sp. mitochondrial metabolism

External pH influences the levels of OXPHOS complexes in *Polytomella* cells grown on ethanol at acidic pH. The levels of respiratory complexes I, III and IV were shown to be noticeably up-regulated at pH 3.7. At pH 3.7, the respiratory rates are higher than at pH 6.0, which is likely the consequence of a higher content in respiratory complexes and also in cytochrome *c* (8). The ratio between the respiratory complexes and the F_oF_1-ATPase is clearly affected by external pH, indicating an influence of pH on core energy metabolism in the colorless alga *Polytomella*.

In amitochondriate protists, ADHE is a cytosolic enzyme that is integral to maintaining redox balance via the NAD(P)H-dependent reduction of acetyl-CoA to ethanol, which is excreted as an end product (34, 36, 37, 46). In *E. coli*, the *adhE* promoter is regulated in response to the cytosolic NADH/NAD ratio (41) also suggesting a role in redox balance in that organism. Both OXPHOS complexes and ADHE in *Polytomella* sp. cells grown on ethanol show a pH-dependent expression and accumulation. The expression of ADHE is higher at moderately acidic pH, using either acetate or ethanol as a carbon source (Fig. 4). The current data are compatible with the view that *Polytomella* mitochondrial ADHE could be involved either in the maintenance of redox balance (ethanol production) or in ethanol assimilation (producing acetyl-CoA and NADH for respiration), or both, depending upon environmental conditions.

Evolutionary considerations

ADHE genes were found in the genomes of several Gram-positive bacteria belonging to the categories bacilli and clostridia among the Firmicutes, in several γ-proteobacteria (particularly enterics), in one actinobacterium and one cyanobacterium, in addition to the previously

characterized sequences from several amitochondriate protists (31, 32, 47) (Fig. 5). Previous phylogenetic analyses have suggested that the anaerobic eukaryotes *E. histolytica* and *G. intestinalis* acquired the gene for their ADHE enzyme through independent lateral gene transfers, possibly from Gram-positive donors (31, 47, 48). The similarity of ADHE sequences from the diplomonads *G. intestinalis* and *S. barkhanus* suggests the presence of the ADHE gene in their common ancestor (47), but an independent origin of *E. histolytica* ADHE seems to be the easiest explanation (33, 47, 48)(see also Fig. 5a). The sequences from *Polytomella* sp. and *C. reinhardtii* mitochondria make the picture somewhat more complicated because they cluster close to, but not with the sequences from *Giardia*, *Spironucleus*, and *Mastigamoeba*.

Notably, the clostridial (low-GC Gram positives) sequences cluster much more closely to the homologues from enterics than they do to the homologues from bacilli (low-GC Gram positives) (Fig. 5). The central branch or split (marked by an asterisk in Fig. 5) separates available ADHE sequences into two larger groups. The overall picture of ADHE sequence similarity is highly reminiscent of that found for pyruvate kinase (PK), where two clusters (I and II) and skew distribution were also observed (49). Schramm *et al.* (49) noted that the PK dichotomy correlated with allosteric properties of the enzymes, not with phylogeny. Although we were unable to identify in the alignment specific motifs or in the literature regulatory properties of ADHE that might distinguish sequences above and below the asterisk in Fig. 5, a pattern of sequence similarity that is largely driven by functional aspects rather than by neutral evolution cannot be excluded *a priori* for this sequence sample. Even if we accept lateral gene transfer from Gram positive donors for the origin of the *Bifidobacterium*, *Pasteurella*, and *Thermosynechococcus* genes, it is difficult to evoke either lateral gene transfer (from what donor?) or ancient duplication and differential loss (too many) to account for the differentness of ADHE from clostridia and bacilli.

Perhaps more caution is warranted when it comes to evidence for horizontal gene transfer on the basis of an unusual phylogeny of an ancient enzyme, as is the case for ADHE. Methods of phylogenetic reconstruction used in this and prior studies to construct the phylogenies from which horizontal gene transfers for ADHE can be inferred are based upon the rates across sites (RAS) models of protein evolution. But we know of no evidence to indicate that proteins in general or ADHE in particular actually evolve according to a RAS model. From the standpoint of molecular evolutionary theory, RAS models have been argued to be less realistic than covarion models (50, 51) and protein evolution simulations taking into account protein folding produced results highly compatible with a covarion model (52, 53). If the model under which a phylogeny is reconstructed deviates strongly from the process by which the protein evolved, the phylogeny can be severely in error (50).

The neighbornet graph of the ADHE protein sequence similarity shown in Fig. 5b indicates that the ADHE data is non-treelike in many respects. This could be due to convergence, noise, or other conflicting signal (25). In a neighbornet graph such conflicting signals in the data become visible that are not represented in purely bifurcating trees. Notwithstanding very complicated patterns of sequence similarity, eukaryotic ADHE, like most enzymes of eukaryotic core energy metabolism studied to date lacks obvious archaebacterial homologues and thus appears to be of eubacterial origin.

Functional considerations

Earlier biochemical studies of *Chlamydomonas* and related green algae had provided evidence for ADHE activity in the mitochondria of these algae (54-56), but until now ADHE sequences were only available for the enzyme from the cytosol of anaerobic eukaryotes with an energy metabolic pattern designated as Type I (57). The expression of *Polytomella* ADHE under aercbic conditions (Fig. 4) extends the occurrence and expression of this enzyme to aerobic eukaryotes growing under aerobic conditions. In *Polytomella* ADHE is clearly localized in mitochondria, extending the occurrence of the enzyme to oxygen-respiring mitochondria as well. The presence of a seemingly anaerobic-specific enzyme in an oxygen-respiring mitochondrion is

not without precedent, because *Euglena* mitochondria contain pyruvate:ferredoxin oxidoreductase, an oxygen-sensitive enzyme otherwise typical of hydrogenosomes (58). Furthermore, the oxygen-sensitive assembly of Fe-S clusters occurs in the mitochondrial matrix (59), perhaps because it is the most oxygen-poor compartment in aerotolerant eukaryotes.

In *E. coli*, ADHE harbors an additional enzymatic activity, that of a pyruvate formate-lyase (PFL) deactivase (39). This is noteworthy because PFL activity has been measured both in mitochondria of *Chlamydomonas* (56) and in whole cells of the green alga *Chlorogonium* (55). PFL also occurs in chytridomycete fungi, where it is localized in hydrogenosomes (60) and evidence for the presence of ADHE in PFL-possessing chytrids has been noted (61). PFL also requires an activating enzyme in *E. coli* (62), and all sequenced prokaryotic genomes surveyed here (underlined in Fig. 5b) that possess ADHE also possess both PFL and PFL activase. Database searching indicated the presence of a gene encoding a PFL-activase in the green alga *C. reinhardtii* (W.M., A.A., unpublished results).

The presence and expression of ADHE in eukaryotes that can live under fully aerobic conditions and that possess fully developed mitochondria is distinctly at odds with the view that eukaryotes acquired ADHE genes specifically as adaptations to an anaerobic lifestyle (47), because *Polytomella* ADHE is expressed under ambient oxygen levels in an oxygen-respiring organelle. Chlorophycean algae are widely distributed in nature and undergo intimate interactions with other organisms. For example, *Chlamydomonas* sp. cells can be parasitized by chytrid fungi (63), and some species of *Chlamydomonas* are endosymbionts of large miliolid foraminifera (64). Thus there has been ample opportunity during evolution for these oxygen respiring algae to have acquired their genes for ADHE via horizontal transfer from yet unidentifiable donors, but the present data are incompatible with the view that if such acquisitions occurred, they did so as an adaptation to an anaerobic lifestyle.

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Abbreviation footnote: ADHE, aldehyde/alcohol dehydrogenase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; BN-PAGE, blue native polyacrylamide gel electrophoresis; Fe-ADH, iron-dependent alcohol dehydrogenase; MTS, mitochondrial targeting sequence; OXPHOS, oxidative phosphorylation.

Data deposition: the sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank (accession number AJ495765).

FIGURE LEGENDS

Fig. 1. **Identification of mitochondrial protein complexes from *Polytomella* sp. using BN-PAGE. (A)** 5-15% BN-PAGE of mitochondrial proteins from bovine heart (beef) (400 µg) and from *Polytomella* sp. cells grown on ethanol at pH 6.0 (P.s.) (600 µg). The most prominent bands of bovine mitochondria correspond to the OXPHOS components: complex I (NADH:Q oxidoreductase), complex II (succinate:Q oxidoreductase), complex III (QH_2:cyt *c* reductase), complex IV (cytochrome *c* oxidase) and complex V (F_oF_1-ATP synthase). The apparent molecular masses of the bovine protein complexes are from (9, 27). **(B)** Identification of *Polytomella* sp. major OXPHOS complexes. NADH-NBT: a BN-PAGE lane was incubated in the presence of NADH and nitroblue tetrazolium; *, indicates the protein complexes that exhibit NADH dehydrogenase activity. Immunoblots: BN-PAGE lanes were transferred to nitrocellulose and probed with the following antisera: **b-F_1**, against subunit β of bovine F_oF_1-ATPase; **core I**, against *N. crassa* core I; and **COXIIA**, against *Polytomella* sp. COXIIA subunit. The apparent molecular masses of *Polytomella* sp. respiratory complexes were estimated using the bovine OXPHOS complexes as markers.

Fig. 2. **(A) BN-PAGE analysis of mitochondria isolated from *Polytomella* sp. cells grown on ethanol at different pH.** Protein complexes from mitochondria (800 µg) isolated from cells grown on ethanol at pH 6.0 and pH 3.7 were separated on a 5-12% BN-PAGE and stained with Coomassie blue R. **(B) Two-dimensional resolution of *Polytomella* sp. mitochondrial protein complexes.** BN-PAGE lanes (Fig. 2A) were cut and placed horizontally for subsequent resolution of the protein complexes into their respective subunits on Tricine-SDS-gel (15% acrylamide). 2D-SDS-PAGE were stained with Coomassie Brilliant blue R250. I, III, IV, V refers to the OXPHOS complexes. Oblique arrows point to the 85-kDa protein (ADHE) whose accumulation is pH-dependent.

Fig. 3. **2D-SDS-PAGE analysis of the soluble mitochondrial protein complexes of *Polytomella* sp.** Left panel, mitochondria from cells grown on ethanol at pH 6.0 were fractionated into their soluble and membrane-bound components and all the fractions were analyzed on BN-PAGE. **Mt**, mitochondria (800 µg); **Mb**, membrane-bound proteins (800□g); **Sol**, soluble proteins (700 µg). The position of the protein complexes as identified in Figure 1 is indicated. •, indicates the position of the 200-kDa complex. Right panel, BN-PAGE lane of the soluble fraction was transferred horizontally on a SDS-gel (12% acrylamide). The protein spots subjected to Edman degradation are pointed with an arrow. The determined N-terminal sequences are reported in Table 1.

Fig. 4. **RNA analysis of ADHE levels in *Polytomella* sp.** Total RNA was isolated from *Polytomella* sp. cells grown on acetate, pH 6.0 (**1**), ethanol, pH 6.0 (**2**) and ethanol, pH 3.7 (**3**). Equivalent amounts of RNA in each lane (15 µg) were hybridized with PCR amplification product (p*Aad*) and *Polytomella agilis* β-tubulin 1 gene (*Tub B1*).

Fig. 5. **Sequence similarity among ADHE proteins.** Open circles indicate branches (or splits) found in >95/100 bootstrap replications. Eubacterial classifications recognized at http://www.ncbi.nlm.nih.gov/Taxonomy/ are indicated. Eukaryotic sequences are indicated in boldface type. Underlined species names indicate that the genome sequence is available at http://www.tigr.org/. Scalebars indicate 0.1 substitution per site. a) Neighbor-joining tree of protein logdet distances. b) NeighborNet graph of protein logdet distances showing multiple conflicting signals. Splits are represented as parallel lines.

Spot number*	Mass (kDa) †	N-terminal sequence	Assignment§
1	85	AAPAAEQKSKSDEEGLSSLKSTLNKAVAAS IS1 : TCGLIAHDPISGYSK IS2 : DLSREALTQIFDALPSADAEK	ADHE
2	60	Blocked	n.d.
3	37	AGx(N,T)(Q,V)AI(G,L)I(N,T)RF(A,G)RIS	n.d.
4	60	ATKEMRFGQD(A,V)RE(R,E)VLQ	HSP60
5	70	ADEVIGIDLVTTNS	HSP70
6	45	SSxTDLKKTVAELIPAEQDR	Citrate synthase
7	31	GSSSGEVGRKVTVLGAAGGIxQPL	Malate dehydrogenase

Table 1. **N-terminal sequences of *Polytomella* sp. soluble mitochondrial proteins.** x, indicates amino acids which were not identified. Residues in parenthesis indicate simultaneous detection. IS1 and IS2 are two internal tryptic fragments of ADHE.
* Numbers of the proteins in SDS-PAGE as indicated in Fig. 3.
† Molecular masses [kDa] estimated from SDS-PAGE in Fig. 3.
§ Assignment made on the basis of sequence similarity with known proteins, except for ADHE, which was identified from the corresponding cDNA sequence (see text).

Additional figure supplied for reviewers only

Figure A. **Multiple sequence alignment of *Polytomella* sp. ADHE with homologues from various sources.** Sequences are from *Polytomella* sp. (*Ps*) ADHE (this work), *C. reinhardtii* (*Cr*) ADHE (this work), *Escherichia coli* (*Ec*) ADHE (P17547); *Entamoeba histolytica* (*Eh*) ADH2 (Q24803); *Clostridium acetobutylicum* (*Ca*) ADHE (P33744) and *Thermosynechococcus elongatus* strain BP-1 (*Te*) ADHE (BAC07780). The cleavable mitochondrial targeting sequence in *Polytomella* ADHE is underlined. Amino acid sequences of *Polytomella* sp. ADHE determined by Edman degradation are in bold and underlined. Conserved patterns in the CoA-dependent ALDH domain and in the Fe-ADH domain are indicated in bold. ., Cys nucleophile in the catalytic center that is invariant in all CoAdependent and CoA-independent ALDH; ., acidic residue in *E. coli* ADHE (Glu-568) that is believed to be necessary and sufficient for aerobic protein inactivation (38). The position of iron-containing ADH signature 1 (PS00913; ADH IRON 1) and signature 2 (PS00060; ADH IRON 2) are indicated. Note the absence in *Polytomella* sp. ADHE sequence of the His residues in the sequence corresponding to Fe-ADH signature 2. NBS, potential nucleotide binding site.

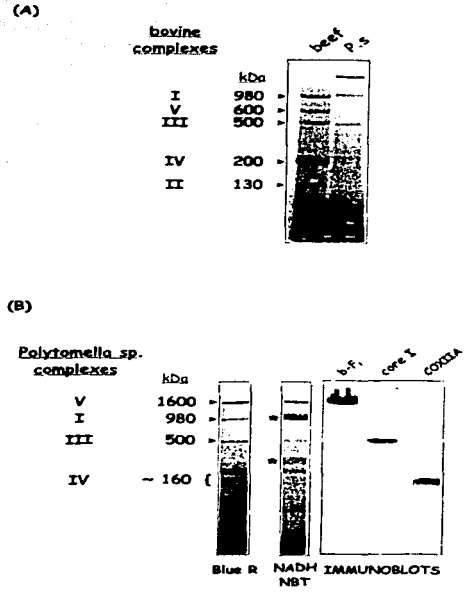


Figure 1

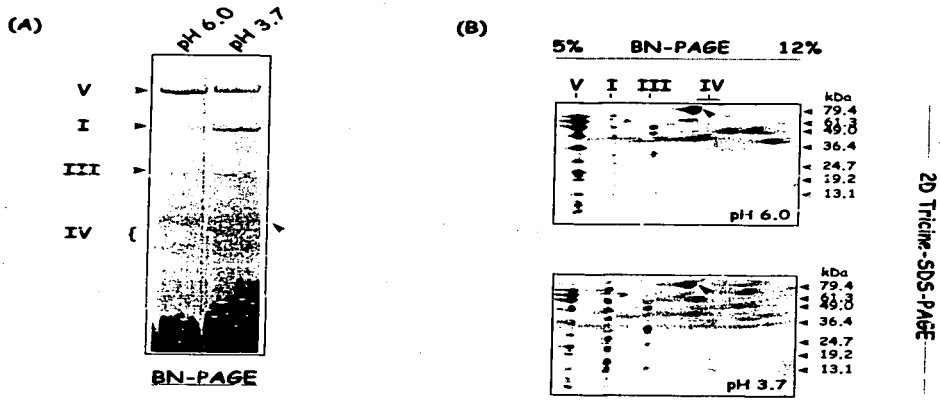


Figure 2

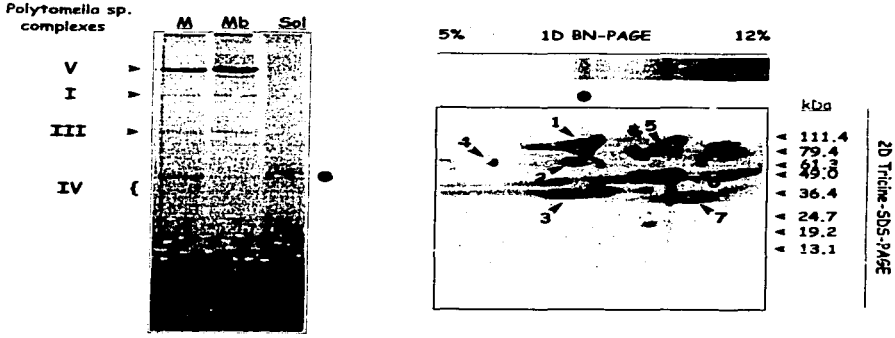


Figure 3

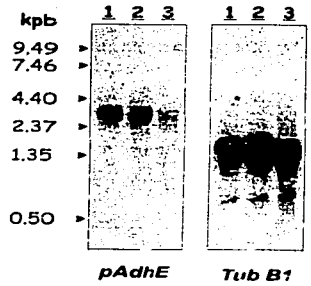


Figure 4

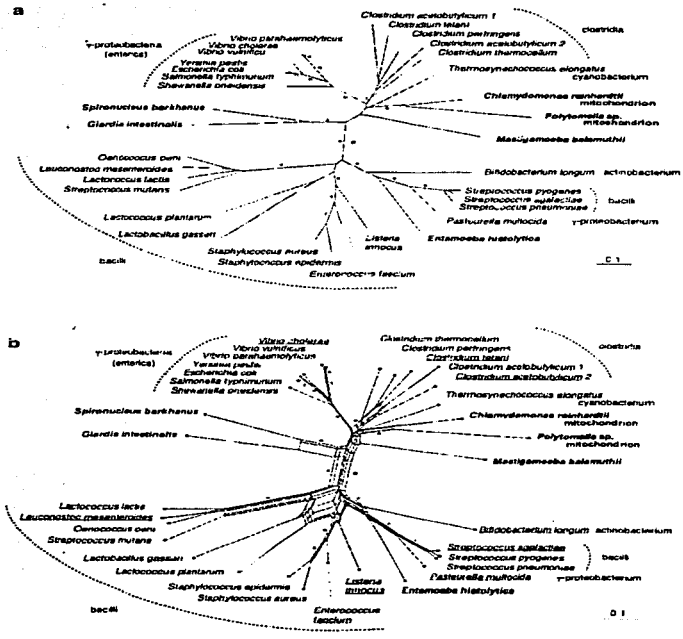
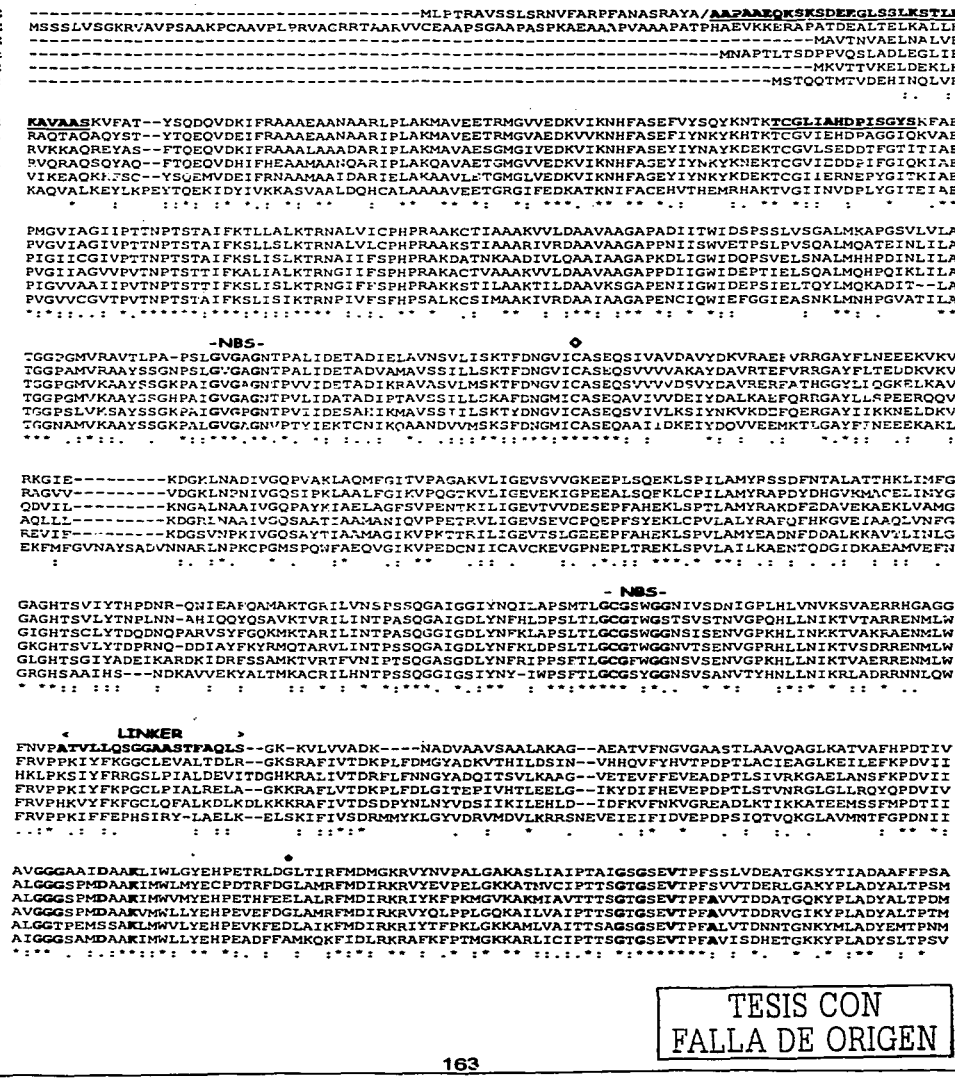
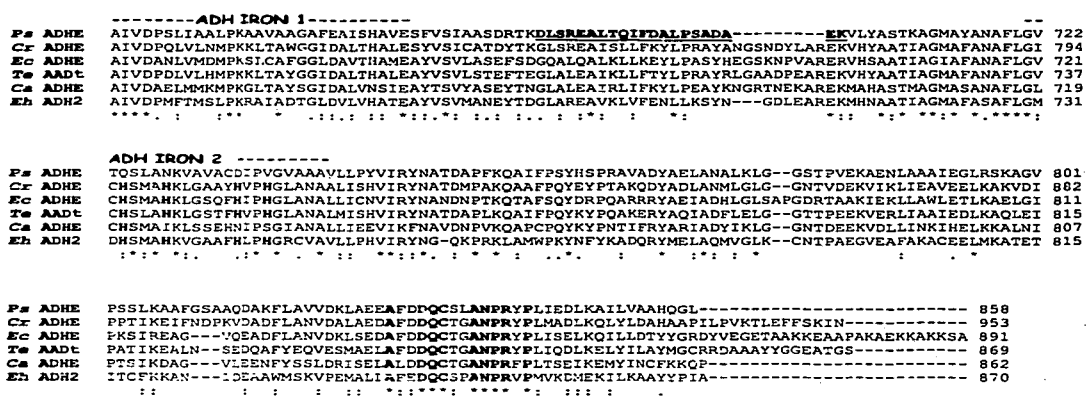


Figure 5





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ARTÍCULO VI : REVISIÓN

Redox-mediated light regulation of mitochondrial function and biogenesis in plants and green algae

Robert van Lis and Ariane Atteia

Sometido a Photosynthesis Research

MITOCHONDRIAL LIGHT REGULATION
Minireview

REDOX-MEDIATED LIGHT REGULATION OF MITOCHONDRIAL FUNCTION AND BIOGENESIS IN PLANTS AND GREEN ALGAE

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Abstract

In photosynthetic cells, mitochondrial respiration is of major importance not only in the dark but also in the light. Important progress has been achieved in our understanding of the roles played by mitochondria in light. The light signal is likely to reach cellular compartments such as the mitochondrion and the nucleus via various chloroplast-originated redox messages. The possible involvement of a redox-mediated light regulation of mitochondrial biogenesis and activity is discussed in view of the available experimental data.

Key words : Chloroplast, electron transport, light regulation, mitochondrion, photosynthesis, redox regulation, reducing equivalents

Abbreviations : AOX, alternative oxidase; CS, citrate synthase; cyt path, cytochrome pathway; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHAP, dihydroxyacetone 3-phosphate; DTT, dithiothreitol; ETC, electron transport chain; GDC, glycine decarboxylase; LEDR, light-enhanced dark respiration; LHC, light-harvesting complex; MDH, malate dehydrogenase; ME, NAD^+-dependent malic enzyme; NADP-MDH, $NADP^+$-dependent malate dehydrogenase; OAA, oxaloacetic acid; PDC, pyruvate dehydrogenase complex; PGA, 3-phosphoglycerate; PQ, plastoquinone; PSI/II, Photosystem i/ii; ROS, reactive oxygen species; Rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase; PS-ETC, photosynthetic electron transport chain; UQ, ubiquinone.

Introduction

For photosynthetic organisms, light is a source of energy but also constitutes a source of information about their environment. In eukaryotic photosynthetic cells, the capture of light by the photosystems in the chloroplast leads to the transport of electrons along a cascade of redox components and results in the production of ATP and the reducing equivalents ferredoxin, NADPH and thioredoxin. The redox state in the chloroplast - as determined here by both the level of reduction of the electron transport chain and the level of reducing equivalents- is enhanced according to the light intensity (Scheibe 1991). An increasing number of studies indicate that light exerts a control on the regulation of gene expression via the redox state (Danon and Mayfield 1994; Allen et al. 1995; Pfannschmidt et al. 1999b) and also influences the efficiency of photosynthesis (Barber and Andersson 1992; Wollman 2001). *In vivo* regulation via changes in the thiol/disulfide state has been strongly implicated in the light-dependent modulation of chloroplast enzyme activities, including a number of enzymes that are part of the Calvin cycle (Buchanan 1991; Scheibe 1991) and of translation factors of chloroplast mRNAs (Danon and Mayfield 1994; Kim and Mayfield 1997). Different components of the photosynthetic electron transfer chain are known to be involved in light-dependent redox regulation, for example the plastoquinone (PQ) pool (Escoubas et al. 1995; Pfannschmidt et al. 1999a) and thioredoxin, as part of the well-characterized ferredoxin-thioredoxin system (Buchanan 1991; Scheibe 1991).

In photosynthetic cells, mitochondrial respiration is of major importance both in the light and in the dark. The mitochondrion is necessary to optimize photosynthetic metabolism under a variety of environmental conditions (Krömer 1995; Gardeström and Lernmark 1995; Hoefnagel et al. 1998; Padmasree et al. 2002). Oxidative phosphorylation is believed to benefit photosynthesis by balancing the cellular energy and redox status. For example, the selective inhibition of the mitochondrial ATP synthase by oligomycin results in the partial inhibition of photosynthesis in illuminated pea leaves (Krömer et al. 1988). Evidence has also been provided that mitochondrial respiration in the light is engaged in the dissipation of excess photoreductants (Raghavendra et al. 1994; Krömer 1995; Padmasree and Raghavendra 1999; Igamberdiev et al. 2001a, b). Products of recent photosynthetic activity such as glycine, malate, oxaloacetic acid (OAA) or NAD(P)H can contribute directly or indirectly to mitochondrial respiration.

Light is a very important environmental signal in photosynthesis because of its major impact on the chloroplast redox state. As will be detailed below, light also exerts a control on gene expression and on enzyme activity in mitochondria. Major clues about the mechanisms involved in the mediation of light regulation occurring in mitochondria are still lacking; whereas the light activation of chloroplast enzymes and translational factors by redox poise been extensively studied, such is not the case for mitochondrial enzymes in photosynthetic cells. Nevertheless, since the functional and structural properties of the chloroplast and mitochondrial electron transfer chains are similar, it is imaginable that mechanisms of redox regulation in the chloroplasts are also applicable to mitochondria. In this review we will summarize the current knowledge on the role played by mitochondria in photosynthesis. Data have been gathered on mitochondrial light- and redox regulation and on the various ways in which the light signal can reach the mitochondrion via redox messages (Figure 1), in an attempt to assess the role of the redox state in mitochondrial light regulation.

Light generates reducing power - in excess

Light (400-700 nm) captured by the light-harvesting complexes in the chloroplast leads to the transport of electrons from water to the final electron acceptors along a cascade of redox components. The most usual electron transport pathway is non-cyclic and results in the reduction of $NADP^+$ by ferredoxin:$NADP^+$ oxidoreductase or of thioredoxin by ferredoxin-thioredoxin reductase. The amount of ATP produced by the chloroplast ATP synthase during photosynthetic linear electron transport to $NADP^+$ is still the subject of debate. Non-cyclic electron transport may result in an ATP/NADPH ratio that ranges from 1.0 to 1.5, while for carbon dioxide fixation in the Calvin cycle an ATP/NADPH of 1.5 or more is needed (Scheibe 1991; Hoefnagel et al. 1998). A tight regulation of chloroplast redox poise is necessary to meet the current demands for energy and reducing equivalents, and to prevent damage of the photosynthetic electron-transfer chain (PS-ETC). Different processes that balance the ATP/NADPH ratio in the stroma have been described. The ATP/NADPH ratio can be balanced by export of NADPH from the stroma to the cytosol. Since NADPH cannot cross the membrane directly, it must be transported via shuttle systems. NADPH can be exported to the cytosol either in the form of malate by the combined action of the $NADP^+$-specific malate dehydrogenase (NADP-MDH) (Scheibe 1987) and dicarboxylates transporters (Hatch et al. 1984; Heineke et al. 1991), or via the phosphate translocator in the form of dihydroxyacetone 3-phosphate (DHAP) (Flügge and Heldt 1991). The process of photorespiration constitutes a redox sink as well and will be addressed below. Proline synthesis from glutamate requires NADPH and was proposed to comprise another redox sink

(Hare and Cress, 1997); under stress conditions, proline can accumulate in the cell. The synthesis of ATP without NADPH production can be achieved via cyclic electron transport (ferredoxin is oxidized by plastocyanin) or via the Mehler pathway, that consists in the ferredoxin-dependent reduction of oxygen to hydrogen peroxide, and the subsequent conversion of hydrogen peroxide to water. According to Krömer (1995), the export of NADPH is preferred over cyclic electron transport to balance the ATP/NADPH ratio. To what extent the processes of balancing the ATP/NADPH occur will mainly depend on the light conditions and the availability of carbon dioxide and nutrients. Finally, chlororespiration is believed to protect the PS-ETC by lowering the reduction state of the PQ pool (Bennoun, 1982; Peltier and Cournac, 2002).

Mitochondria as photosynthetic redox sink

Mitochondrial function in photosynthetic organisms is crucial for optimal chloroplast metabolism. Important roles for mitochondria in photosynthesis include the production of ATP for sucrose synthesis, the supply of carbon skeletons and metabolites for biosynthesis, especially nitrogen assimilation (Krömer 1995; Hoefnagel et al. 1998; Padmasree et al. 2002). The importance of mitochondrial activity in sustaining efficient photosynthetic activity and in preventing photoinhibition is detailed below.

Specific features of mitochondria in photosynthetic cells

The mitochondria of plants and green algae possess a number of additional protein components as compared to mitochondria from mammals and most other organisms. Of specific importance here are the glycine decarboxylase (GDC) complex, which catalyzes the mitochondrial step of the photorespiratory pathway; the alternative rotenone-insensitive NAD(P)H:Q oxidoreductases, which can oxidize either cytosolic or matrix NAD(P)H (Soole and Menz 1995; Møller, 2002); and the alternative, cyanide-resistant oxidase (AOX). The AOX branches from the cyanide-sensitive cytochrome pathway (cyt path) at the level of the ubiquinone (UQ) pool. The AOX enzyme can receive electrons from NADH oxidation at complex I (rotenone-sensitive NADH:Q oxidoreductase) or at the alternative NAD(P)H dehydrogenases, and from $FADH_2$ oxidation at complex II (succinate:Q oxidoreductase). The AOX and the rotenone-insensitive NAD(P)H dehydrogenases do not translocate protons across the mitochondrial inner membrane and will thus not give rise to ATP production. Therefore, the rotenone-insensitive NAD(P)H dehydrogenases allow NAD(P)H oxidation to occur entirely without ATP production if the electrons are subsequently routed to the AOX, which provides photosynthetic mitochondria with an alternative, non-phosphorylating respiratory pathway. This AOX pathway was first proposed to be an energy overflow mechanism to enable respiration to continue under high cytosolic ATP/ADP and NADH/NAD ratios (Lambers 1982). More recently, the AOX pathway was shown to be involved in preventing the formation of reactive oxygen species (ROS) which cause damage to the respiratory chain (Maxwell et al. 1999).

The aforementioned components provide the photosynthetic cell with a high respiratory flexibility and allow the fast and efficient adaptation to changes in environmental conditions, especially light (Gardeström 1996; Mackenzie and McIntosh 1999). As will be detailed later, the expression and activity of these additional enzymes is regulated by light and development.

Mitochondria as part of the photorespiratory pathway

The process of photorespiration involves the oxygenic activity of the ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco) in the chloroplast stroma under carbon dioxide-limiting conditions, and also includes peroxisomal and mitochondrial stages. Glycine that results from the conversion of photosynthetic glycolate in the peroxisome, is imported into the mitochondrial matrix. There it is converted by the GDC complex and the serine hydroxymethyl transferase into serine, with a concomitant reduction of NAD^+ to NADH (Douce and Neuburger 1989; Douce et al. 2001). According to Krömer (1995), about half of the NADH that originates from glycine oxidation is shuttled to the peroxisome while the other half is used for mitochondrial respiration. This way, photorespiration results in a net export of reductants from the chloroplast to the mitochondrion and the peroxisome. Indeed, the mitochondrial NADH/NAD^+ ratio increases during photorespiratory conditions and decreases in the presence of aminoacetonitrile, an inhibitor of the GDC (Wigge et al. 1993). Studies using respiratory inhibitors revealed that in photosynthetic tissues, glycine is preferred to other respiratory substrates in mitochondrial respiration (Dry et al. 1983; Igamberdiev et al. 1997). The electrons from the NADH that is formed during glycine oxidation seem to be preferably routed via the non-phosphorylating respiratory pathway: firstly, in C3 plants, it has been demonstrated that rotenone is relatively inefficient in inhibiting glycine oxidation (Igamberdiev et al.1997, 1998). Secondly, glycine oxidation increased the cytosolic ATP/ADP ratio in the presence but not in the absence of carbon dioxide, which indicates the engagement of the non-phosphorylating pathways under photorespiratory conditions (Igamberdiev et al. 1997). Moreover, it has been shown that in a GDC-deficient barley mutant, photorespiratory conditions lead to an over-reduction and over-energization of the cell, especially in the chloroplast (Igamberdiev et al. 2001a). Photorespiration therefore appears as an energy sink to avoid the over-reduction of the PS-ETC and contributes to the prevention of photoinhibition.

Mitochondria and photoinhibition

Photosynthetic efficiency decreases when green algae and higher plants are exposed to higher light intensities than necessary for normal growth. This phenomenon called photoinhibition is due to the over-reduction of the PS-ETC, causing inactivation of the reaction centers. The importance of mitochondrial respiration in preventing photoinhibition has been demonstrated by different approaches. The susceptibility of the green alga *Chlamydomonas reinhardtii* to photoinhibition was increased when the cells were incubated in the presence of an inhibitor of the cytochrome oxidase pathway (KCN) or in the presence of uncouplers of oxidative phosphorylation (FCCP, CCCP) (Singh et al. 1996). In addition, the complete recovery of photosynthetic capacity after photoinhibition was significantly slower in cells treated with inhibitors of mitochondrial respiration than in untreated cells (Singh et al. 1996). Similarly, in leaf cells, the inhibition of the mitochondrial F_0F_1-ATP synthase by oligomycin enhanced photoinhibition (Krömer et al. 1988; Saradedevi and Raghavendra 1992) and also resulted in an increase in redox status in pea protoplasts (Padmasree and Raghavendra 1999). These data point towards an important function of mitochondria in oxidizing excess chloroplast redox equivalents, which can be used for ATP synthesis. The AOX pathway is likely to play a role in the prevention of photoinhibition since

excess photosynthetic reducing equivalents can be oxidized without ATP production when cytosolic ATP demand and ADP availability are low (Lambers 1985).

It was proposed that mitochondria participate in the prevention of photoinhibition also by oxidizing cytosolic NAD(P)H via the external NAD(P)H dehydrogenases (Raghavendra et al 1994). Export from the chloroplast of malate (via the malate valve) and DHAP (via the phosphate translocator) can result in the production of NADPH in the cytosol (Scheibe 1987; Heineke et al, 1991; Flügge and Heldt 1991). Alternatively, malate itself can be imported into the mitochondria, whereas DHAP can give rise to pyruvate (or malate), also a mitochondrial substrate. Oxidation of cytosolic NAD(P)H but probably more importantly the oxidation of malate, taken together with the utilization of photorespiratory NADH, likely protect the cell from photoinhibition.

Light-enhanced dark respiration

After a period of photosynthesis, the rate of respiratory oxygen consumption immediately following transition to the dark is significantly enhanced (Raghavendra et al. 1994). This phenomenon is known as light-enhanced dark respiration (LEDR) and is commonly observed in plants (Azcón-Bieto et al. 1983; Reddy et al. 1991) and green algae (Beardall et al. 1994; Xue et al. 1996). LEDR is a photosynthesis-dependent phenomenon that is greatly reduced in the presence of DCMU, an inhibitor of the non-cyclic photosynthetic electron transfer (Xue et al. 1996). Igamberdiev et al. (2001b) have shown that during LEDR the levels of malate and citrate as well as the activity of the chloroplast $NADP^+$-MDH in the barley protoplasts decline. In contrast, during LEDR, the activity of the mitochondrial NAD^+-malic enzyme is increased (Igamberdiev et al. 2001b). An increase in the AOX activity also suggests that the non-phosphorylating mitochondrial respiration is involved in LEDR (Azcón-Bieto et al. 1983). The phenomenon of LEDR clearly indicates the rapid response of mitochondria to products of photosynthetic electron transport, especially malate. The period of enhanced mitochondrial respiration after photosynthesis is probably required to reach steady-state substrate levels required for dark metabolism.

Light-modulated regulation in mitochondria

Experimental evidence has been provided that in photosynthetic organisms, light influences the function and biogenesis of the mitochondria.

Light regulation of mitochondrial enzyme activities

In the dark, mitochondria are the only source of ATP for the photosynthetic cell. It is therefore assumed that the phosphorylating cyt path is relatively more active with respect to the non-phosphorylating alternative pathways in the dark than during photosynthesis (Svensson and Rasmusson 2001). The fact that the oxygen consumption in leaves of various plants was found to be up to 3.5-fold higher in the light than in the dark (Padmasree et al. 2002) could be due in part to significant engagement of the alternative pathway, bearing in mind that the cyt path also continues to operate in order to provide ATP, which is necessary for optimal photosynthetic metabolism. As detailed above, the non-phosphorylating pathways are relatively more engaged in

periods of photorespiration. The level of photorespiration generally rises with leaf development, since the increase in photosynthetic capacity leads to a more pronounced effect of light on chloroplast metabolism and redox state. As an example, the activity of the AOX was found to be low in mitochondria from young pea leaves but increased substantially as the tissue matured, which is mostly caused by the increase in photorespiration (Lennon et al. 1995). Moreover, the relative participation of the internal rotenone-insensitive NAD(P)H dehydrogenase was significantly increased with potato leaf development, while the capacity of complex I and of the external NAD(P)H dehydrogenase were unchanged. Also, rotenone-insensitive NADH oxidation was decreased in dark-treated as compared to light-grown plants. The activity of the external NADPH dehydrogenase was significantly higher in the light than in the dark, and likely indicates a photosynthetic contribution to the cytosolic NADPH pool (Svensson and Rasmusson 2001).

The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the oxidation of pyruvate to carbon dioxide and acetyl-CoA, the primary entry point of the TCA cycle. *In vivo* measurements showed a 40-60% inhibition of the PDC activity in pea leaves in the light as compared to darkness (Budde and Randall 1990; Gemel and Randall 1992). A consequence of a decrease in TCA activity is a lower production of NADH, which may constitute a compensation for production of NADH that originates from photosynthetic reductants. Upon illumination of pea leaves, the activity of the mitochondrial PDC drops to a steady state level of less than 25% of the activity measured in the dark. This decrease was not observed under conditions unfavorable for photorespiration, which indicates that the effect of light on mitochondrial function is associated with photosynthesis and photorespiration (Budde and Randall 1990).

Light-dependent expression of components of the respiratory network

Using potato leaves, Svensson and Rasmusson (2001) followed the expression and accumulation of two subunits of complex I, the nuclear-encoded iron-sulfur 76-kDa subunit and the mitochondrially-encoded NAD9 subunit, in parallel with subunits NDA1 and NDB1 of the internal and external rotenone-insensitive NAD(P)H dehydrogenase, respectively. Western-blot analysis showed that the NDA1 protein was detected in mitochondria isolated from light-grown plants while it was undetectable in dark treated plants; the levels of NDA1 were also shown to increase during potato leaf maturation. In contrast, the levels of accumulation of the 76-kDa and NAD9 subunits of potato complex I were comparable in light-grown and dark-treated leaves, and remain rather constant during leaf development. Northern-blot analysis revealed that *nda1* mRNA levels were strictly light-dependent, and correlated with an up-regulation of NDA1 biosynthesis in the light (Svensson and Ramusson 2001).

The expression of cytochrome *b* (*cob*) encoded within the mitochondria was shown to be light-dependent. In the photosynthetic alga *Chlorogonium elongatum*, the number of *cob* gene copies and the level of *cob* mRNA were about 5-fold higher in cells grown under heterotophic conditions than in cells grown under autotrophic conditions (Kroymann et al. 1995). Interestingly, the expression pattern of the *cob* gene in the green alga was opposite to the expression of the chloroplast genes *psbA* and *rbcL*, coding for the D1 protein of PSII and the large subunit of Rubisco, respectively (Kroymann et al. 1995). The accumulation levels of cytochrome *b* in the alga grown under different conditions were not determined.

By Western blot analysis, the AOX was not detected in darkness but could be detected in mature and senescent potato leaves (Svensson and Rasmusson 2001). Etiolated soybean (*Glycine max* L.) seedlings transferred to light showed a strong increase in the levels of Aox2

mRNA. The correlation between the increase in *Aox2* mRNA and the accumulation of AOX protein indicates a transcriptional regulation by light (Finnegan et al. 1997).

Light-dependent expression of mitochondrial components not involved in respiration

The activity of GDC was approximately ten times greater in the mitochondria of greened tissues than in etiolated pea leaves or potato tuber (Walker and Oliver 1986). Studies showed that this increase in activity results from a dramatic increase in the synthesis of the GDC subunits H, L and P (Walker and Oliver, 1986; Macherel et al. 1990) which is controlled at the transcriptional level (Turner et al. 1993). A large body of evidence indicates that light directs the transcription of the GDC subunits in the same way as it does for photosynthetic genes like those encoding the small subunit of the Rubisco (*rbcS*) or the chlorophyll a/b binding protein of the light harvesting complex (*cab*). The expression of *gdcH*, *gdcT* and *gdcP*, the genes that encode respectively the subunits H, T and P, during greening of etiolated *Arabidopsis thaliana* or during pea leaf development follows a transcriptional regulation scheme similar to that of the photosynthetic genes *rbcS* and *cab* (Srinivasan and Oliver 1995; Vauclare et al. 1996). By a combination of deletion and mutagenesis experiments in the promoter region of these nuclear *gdc* genes, *cis*-acting elements have been identified; these DNA sequences are highly similar to the light-responsive motifs found in several light-regulated photosynthetic genes, such as *rbcS* and *cab* genes (Datta and Cashmore 1989; Green et al. 1987, 1988). Therefore, the light-response of the GDC subunits is likely due to the binding in the 5'-untranslated regions of *gdcT* and *gdcH* of light-dependent expression factors (Srinivasan and Oliver 1995; Vauclare et al. 1998)

In potato, the expression of the mitochondrial citrate synthase (CS) was detected in all tissues analyzed. However, the mRNA levels were higher in photosynthetic tissues than in underground heterotrophic tissues (Landschutze et al. 1995). This possibly indicates that CS plays a role in photosynthesis, for example in the export of carbon skeletons that serve in N-assimilation. Furthermore, the mitochondrial NAD^+-dependent malic enzyme (NAD-ME) that catalyzes the conversion of malate into pyruvate, plays an essential role in photosynthetic carbon fixation. In the C4 plant amaranth, the synthesis of the alpha subunit of the NAD-ME and the accumulation of its corresponding mRNA were shown to be strongly light-dependent (Long and Berry 1996).

Redox regulation in mitochondria of photosynthetic organisms

Results of recent investigations are consistent with a critical role of the mitochondrial redox poise in the regulation of mitochondrial biogenesis and activity of photosynthetic organisms. Monitoring the effect of the mitochondrial redox status on mitochondrial function and protein synthesis is difficult to achieve *in vivo* and is therefore usually done with isolated mitochondria.

Redox regulation of the mitochondrial enzyme activities

AOX is the best-studied example of a mitochondrial activity regulated by redox state. Plant AOX exists either as a less active covalently or a more active non-covalently linked homodimer in the mitochondrial inner membrane. The monomer exhibits a cysteine residue on the matrix side that serves as a regulatory sulfhydryl/disulfide site (Umbach and Siedow 1993; Umbach et al. 1994). It was shown that the reduction of tobacco AOX to its more active form was mediated by the oxidation of specific TCA cycle substrates that are linked to the reduction of NAD(P), including isocitrate and malate. Vanlerberghe and McIntosh (1997) have proposed that the reduction of the AOX results from the production of reducing power (NADPH) by the activity of isocitrate dehydrogenase or malate dehydrogenase. Certain □-ketoacids, especially pyruvate, were also found to activate the reduced AOX (Millar et al. 1993; Vanlerberghe et al. 1995). In addition, the AOX activity in soybean cotyledon mitochondria is regulated by the redox poise of the UQ pool (Dry et al. 1989).

The activity of CS was also shown to be sensitive to the redox state. The citrate synthases of potato, pummelo (*Citrus maxima*) and *A. thaliana* are inactivated in crude extracts by the strong oxidizing agent diamine while their activity is greatly stimulated by dithiothreitol (DTT) (Stevens et al. 1997). The PDC in pea leaf mitochondria was found to be partially inhibited by NADH and also by ADP (Moore et al. 1993). Glutathione has been found to influence the activity of DNA topoisomerase in mitochondria isolated from carrot. The activity of this enzyme was increased in the presence of reduced glutathione (GSH), while the oxidized dimer (GSSG) caused inactivation (Konstantinov et al. 2001). These results point to a role for glutathione in mitochondrial redox regulation of DNA transcription or replication.

Redox regulation of gene expression and protein synthesis

An experimental approach that was used to test the possible regulation by redox state of the expression of mitochondrially-encoded proteins, consists in studying the RNA synthesis in model redox conditions, created by the addition of oxidizing and reducing agents to isolated mitochondria. In such a manner, the incorporation of ^{3}H-UTP into RNA in isolated maize mitochondria was found to increase in the presence of 5 mM ferricyanide while it decreased in the presence of 5 mM dithionite (Konstantinov et al. 1995). In potato mitochondria, RNA synthesis was found to depend on the redox poise of the Rieske protein, a subunit of the bc_1 complex that contains an iron-sulfur center as redox group (Wilson et al. 1996). RNA synthesis was diminished by the respiratory substrates malate and succinate but also by addition of DTT, indicating that increasing the reduction of the respiratory chain inhibits RNA synthesis. The addition of antimycin A, an inhibitor of the bc_1 complex that prevents the reduction of the Rieske protein, resulted in an increase of mitochondrial RNA synthesis, whereas KCN, which causes reduction of the chain including the Rieske protein, decreased RNA synthesis. The redox potential at which UTP incorporation occurred was determined by redox titration to be + 270 mV, which equals the redox potential of the mitochondrial Rieske protein. A similar role for the chloroplast Rieske protein, a subunit of the b_6f complex, was proposed earlier to regulate RNA synthesis within the chloroplast (Pearson et al. 1993). In the experiments described above, the incorporation of ^{3}H-UTP reflects the synthesis of any type of RNA within the mitochondria; the mitochondrial genes that are susceptible to redox regulation have not yet been identified.

The addition of antimycin A to tobacco suspension cells caused the levels of *Aox1* mRNA to increase rapidly while mRNA levels for two proteins of the cyt path, cytochrome *c* and subunit I of the cytochrome *c* oxidase were unchanged (Vanlerberghe and McIntosh 1994). The increase in the *Aox1* mRNA level was accompanied by an increased AOX protein level. High reduction levels of the UQ pool, which can be caused by the addition of antimycin A, has been implicated in the control of the AOX (Dry et al. 1989; Hoefnagel and Wiskich, 1998). Moreover, it was shown that hydrogen peroxide plays a role in the induction of AOX gene expression in *Petunia hybrida* cells (Wagner 1995). These *in vivo* observations therefore indicate that the increase of RNA synthesis in response to changes in redox conditions in the cell is not a general phenomenon but appears as a rather specific event. The data also provide an example of a mitochondrial-originated redox signal that reaches the nucleus where it triggers the expression of a nuclear gene that encodes a (plant-specific) mitochondrial protein.

Protein synthesis was also found to be modulated under different model redox conditions. Under oxidizing conditions created by the addition of ferricyanide an inhibition of the protein synthesis in isolated plant mitochondria is observed (Allen et al. 1995a). Also, an inhibitor of complex II (thenoyltrifluoro-acetone or malonate) added alone or in combination with rotenone (complex I inhibitor) and dicumarol (internal rotenone-insensitive NADH dehydrogenase inhibitor) resulted in an inhibition of protein synthesis, as measured by ^{35}S-methionine incorporation (Escobar Galvis et al. 1998). In contrast, when rotenone and dicumarol were added together the protein synthesis was stimulated. Based on these data, it was concluded that a subunit of complex II or a closely-associated redox component might serve as a redox sensor that could be part of the regulation system of the UQ pool. It seems contradictory that under oxidizing conditions (ferricyanide addition), RNA synthesis is increased (see above) whereas protein synthesis is diminished. It was proposed that this difference was due to a differential regulation depending on the level of gene expression (Escobar Galvis et al. 1998).

The participation of glutathione (γGlu-Cys-Gly) in the regulation of protein synthesis in plant mitochondria was suggested by Konstantinov et al. (1998). These authors have shown that the incorporation of [^{14}C]-leucine into protein in mitochondria isolated from maize seedlings increased in the presence of oxidized glutathione while it was significantly repressed in the presence of reduced glutathione. These data could suggest a thiol-mediated redox regulation of mitochondrial protein synthesis.

Light as factor in mitochondrial redox regulation

Molecular responses like transcription, translation, enzyme activation or apoptosis can be regulated via redox control. Redox regulation of a given biological process can be typically defined as the control exerted by the redox state of some constituent molecules on the process (Pfannschmidt et al. 2001a). In photosynthetic organisms, many redox responses are a consequence of light: light initiates photosynthetic electron transport that subsequently gives rise to various redox signals; in turn, these signals regulate a number of biological processes. In the following, we summarize the current understanding on the redox-mediated light regulation of photosynthetic activity and chloroplast biogenesis. Then, the possibilities of how light-induced redox messages can reach the mitochondria and influence mitochondrial function or biogenesis are evaluated.

Mechanisms of redox-mediated light regulation in chloroplasts

Our knowledge on the redox-mediated light regulation of the activity and biosynthesis of the photosynthetic apparatus is growing. At present, probably the best-understood mechanism of redox control in chloroplast is the ferredoxin-thioredoxin system. In the light, ferredoxin is reduced as a consequence of photosynthetic electron transport and can then be used by ferredoxin-thioredoxin reductase to produce reduced thioredoxin. This is illustrated by the level of reduction of thioredoxin that has been reported to be of 0-20% in darkness and up to 90% in light (Scheibe 1981; Crawford et al. 1989). The ferredoxin-thioredoxin system in chloroplasts serves to modulate or maintain the thiol/disulfide redox state of specific proteins in response to light and other environmental factors. This system provides the chloroplast with means to coordinate the activities of various photosynthetic processes. The ferredoxin-thioredoxin system is involved in the activation of a number of key chloroplast enzymes, such as the enzymes of the Calvin cycle (Scheibe 1991; Buchanan 1991) or the NADP-MDH (Ruelland and Miginiac-Maslow 1999). In light, the activity of these enzymes is enhanced due to the reduction of regulatory disulfide bonds by reduced thioredoxins. Also, in plant cells and green algae, a great increase in synthesis rate of specific chloroplast proteins is observed as a result of the activation of specific translational factors by reduced thioredoxins. Reduction of the disulfide bonds of the RNA-binding protein complex enables it to bind to chloroplast mRNA, thereby allowing its translation. A well-known example is that of *psbA* mRNA which encodes the D1 protein of PSII in the unicellular alga *C. reinhardtii* (Danon and Mayfield 1994).

Redox-controlled phosphorylation of thylakoid membrane proteins represents a unique system for the regulation of light energy utilization in photosynthesis and controls, for example, the distribution of excitation energy between the photosystems I and II (state transition) as well as the photosystem stoichiometry (Pfannschmidt et al. 1999b; and references therein). Light-activated thylakoid protein phosphorylation is in great part regulated by the redox state of the PQ pool. Light induces phosphorylation of PSII proteins (D1, D2) by activating protein kinase(s) via reduction of PQ (Allen et al. 1995b; Pfannschmidt et al. 1999b). In addition, protein phosphorylation of the PSII subunits was strongly influenced by thiol disulfide redox state, suggesting that the ferredoxin-thioredoxin system is involved as well (Carlberg et al. 1999). Furthermore, the cytochrome b_6f complex is recruited for redox sensing and signal transduction (Wollman 1999). Subunit V of *C. reinhardtii* b_6f complex is reversibly phosphorylated upon state transition of the light-harvesting antennas; this subunit is proposed to play a role in the signal transduction during redox controlled adaptation of the photosynthetic chain (Hamel et al. 2000). Recently, a novel plant-specific protein of 9-kDa named thylakoid soluble protein (TSP9) was identified and shown to undergo light-dependent phosphorylation followed by a partial release from the thylakoid membrane (Carlberg et al 2003). Previously, TSP9 was known as a thylakoid membrane associated 12-kDa phosphoprotein which phosphorylation was redox state dependent (Bhalla and Bennet, 1987; Cheng et al. 1994). Reversible protein phosphorylation possibly occurs as part of a two-component redox regulatory system, similar to that found in bacteria and cyanobacteria (Stock et al. 1989; Allen 1993).

Evidence has been provided for a coupling between the redox state of the PQ pool and the regulation of transcription of a number of chloroplast genes (Pfannschmidt et al. 1999a; Tullberg et al. 2000) as well as nuclear genes (Pfannschmidt et al. 2001b). For example, in the green alga *Dunaliella tertiolecta*, the expression of the nuclear *cab* genes that encode the chlorophyll a/b binding protein is up-regulated in conditions that reduce the PQ pool (Escoubas et al. 1995). In *Arabidopsis*, the PQ redox state was also shown to be involved in the transcriptional

regulation of the nuclear gene encoding the cytosolic ascorbate peroxidase (Karpinski et al 1997). Also hydrogen peroxide was shown to be involved in the induction of the ascorbate peroxidase in excess light (Karpinski et al. 1999). Indeed ,photosynthetic electron transport generates ROS including hydrogen peroxide, which is thought to be a mediator of the redox state within the cells (Noctor et al. 2000). Glutathione is a ubiquitous tripeptide that is involved in quenching the generated ROS through the ascorbate/glutathione cycle (Alscher 1989; Smirnoff 1995). Noctor and Foyer (1998) suggested that the ratios of the reduced and oxidized forms of antioxidants such as ascorbate and glutathione can have an important signaling function in the regulation of gene expression. The chloroplast RNA polymerase from *Sinapis alba* for example shows control of transcription by an associated protein kinase that responds to reversible thiol/disulfide formation mediated by glutathione (Baena-Gonzalez et al. 2001).

Light signal transduction via redox state for regulation of mitochondrial function and biogenesis

In light, mitochondria from photosynthetic organisms are critical in optimizing the photosynthetic activity by maintaining the redox and energy balance in the cell. So far, the mechanisms that control the biogenesis and activity of the mitochondria in light are largely unknown. Photosynthetic activity generates multiple redox signals which, as detailed above, elicit a high level of control on chloroplast activity and gene expression. In analogy to the redox-mediated light regulation in chloroplasts, we suggest that a key mechanism for the light-response of mitochondrial activity and biogenesis involves chioroplast-originated redox signals and metabolites. Redox signals produced by photosynthesis can be considered light signal-transducing molecules that relay information on the chloroplast redox state since they can be exported to different cell compartments (Figure 1). As said earlier, the export of reducing equivalents from the chloroplast to the mitochondria occurs through different mechanisms, including photorespiration, the malate valve and the phosphate translocator (DHAP/PGA); the imported reducing equivalents influence the mitochondrial redox state -notably the $NADH/NAD^+$ ratio and the reduction state of the respiratory chain- which will in turn have regulatory consequences. Redox messages may also be transmitted throughout the cell via hydrogen peroxide, whereafter the signal of hydrogen peroxide can be relayed indirectly via redox-transduction mechanisms involving antioxidants (glutathione/ascorbate), or via a two component-like redox regulation. Also the cellular adenylate status depends on the photosynthetic conditions and will provoke adaptive responses that involve electron partitioning of the respiratory chain.

In light, a massive flow of carbon passes through the GDC complex in the mitochondrial matrix, giving rise to large amounts of NADH. About half of the NADH produced by photorespiration will be reoxidized in the mitochondrion (Krömer, 1995), mainly via non-phosphorylating pathways (Igamberdiev et al. 2001a; Rasmusson and Svensson 2001). The fact that NADH resulting from glycine oxidation is preferentially utilized by the alternative respiratory pathway may suggest a substrate channeling between GDC and the alternative NAD(P)H dehydrogenases and/or a specific light-dependent down-regulation of the cyt path (see below). Besides photorespiration, other chloroplast-originated reducing equivalents can be imported into the mitochondria and modulate the NADH/NAD ratio (Figure 1). When photosynthetic reductants are oxidized by the respiratory chain, it is assumed that the resulting reduction level of the chain, and in particular of the UQ pool, exerts a control on the regulation of mitochondrial metabolism by similar mechanisms of redox regulation as for the PQ pool in the chloroplast. The UQ pool makes

a good sensor of the redox state of the respiratory chain, since the electrons from all the different pathways are first passed on to ubiquinone. The reduction state of the UQ pool determines the engagement of the AOX (Dry et al. 1989; Hoefnagel and Wiskich, 1998) and possibly of other enzymes. Photorespiratory NADH for example could trigger the activation of the AOX by increasing the reduction state of the UQ pool. Other components of the respiratory chain such as the Rieske protein have been indicated as redox sensors (Wilson et al. 1996), possibly as part of a two component redox regulation system in mitochondria, as envisioned by Allen (1993; Allen and Raven, 1996). Recently, nuclear genes for 'bacterial' histidine sensor kinases and aspartate response regulators that seem to be targeted to mitochondrial membranes have been identified in *A. thaliana* and *Zea mays* (Forsberg et al, 2001). Earlier, Håkansson and Allen (1995) found histidine and tyrosine phosphorylation in pea mitochondria as evidence for protein phosphorylation in respiratory redox signaling. Such redox signaling likely regulates gene expression within the mitochondria but may also regulate nuclear gene expression.

The activity of the pyruvate dehydrogenase complex (PDC) in the mitochondria is down-regulated by light (Budde and Randall, 1990) and with development of photosynthetic capacity (Luethy et al., 2001). The activity of the PDC is known to be controlled by several interacting mechanisms, notably reversible protein phosphorylation and product inhibition (Randall et al. 1989; Moore et al. 1993). The inactivation of the enzyme that is observed with increasing photosynthetic activity could be due to product inhibition by the NADH of photorespiratory origin. It was indeed reported that the inactivation of the PDC by phosphorylation in the light was relieved by conditions of reduced photorespiration whereas it was enhanced by NADH and NH_4^+, products of glycine decarboxylation (Padmasree et al., 2002 and references therein). The light-regulation of PDC is of importance since the enzyme occupies a unique position at the interface of several metabolic pathways, controlling the mitochondrial carbon flow.

In light conditions, the activity of the AOX and of CS dramatically increases. The increased activity is the result of an enhanced synthesis of the proteins but also of a stimulation of the enzyme activity. For example, full activation of the AOX enzyme requires the presence of pyruvate (Millar et al. 1993; Vanlerberghe et al. 1995), which levels increase with the light inhibition of the PDC. Furthermore, for high activity in light, the mitochondrial AOX and CS require reduction of cysteine disulfide groups (Stevens et al. 1997; Rhoads et al. 1998). Due to the presence of regulatory sulfhydryl/disulfide sites, the AOX and CS are proposed to be potential targets of a flavoenzyme NADPH thioredoxin reductase (Rhoads et al. 1998; Laloi et al. 2001) which has been recently identified in plant mitochondria (Laloi et al. 2001). In the case of plant AOX, the reducing power (NADPH) generated by the oxidation of certain TCA-cycle substrates, especially malate and isocitrate, has been proposed to mediate the reduction of the AOX dimer by mitochondrial thioredoxin (Vanlerberghe et al. 1995). The mechanism that leads to the reduction of the plant CS disulfide bonds and thus to enzyme activation is not known. The fact that animal citrate synthases exhibit the same cysteine residues as plant citrate synthases but are not redox-sensitive (Stevens et al. 1997) suggests that an additional, possibly light-associated level of control is necessary in photosynthetic organisms.

The expression of a number of nuclear genes for mitochondrial proteins increases upon illumination of plant leaves, indicating that the light signal is able to reach the nucleus. An identical expression profile is observed between the H- and T-protein subunits of the GDC and several nuclear genes for chloroplast proteins -like the *cab* and *rbcS* genes. In the case of the nuclear *cab* gene, its expression is regulated by the redox state of the PQ pool (Escoubas et al. 1995) likely via a two-component redox system. It is conceivable that a redox-dependent regulation via, for example, the PQ pool can also coordinate the expression of nuclear genes for

mitochondrial proteins required to optimize photosynthetic activity, such as the GDC subunits. Other light-transduced redox signals that originate in the chloroplasts, such as those associated with hydrogen peroxide and antioxidant cycles (the ascorbate/glutathione cycle), may be distributed throughout the cell (Foyer et al. 1997) and influence the gene expression of mitochondrial proteins. In that respect, it has been shown that hydrogen peroxide plays a role in the induction of AOX gene expression in *Petunia hybrida* cells (Wagner 1995). Hydrogen peroxide may relay redox signals via systems that respond to hydrogen peroxide itself or via systems that depend on the redox state of glutathione and ascorbate. Evidence has been provided for the presence of the ascorbate-glutathione cycle in mitochondria of pea leaves (Jimenez et al. 1997). Moreover, in high light-exposed *Arabidopsis* leaves, hydrogen peroxide is involved in the expression of the nuclear *apx*1 and *apx*2 gene, coding for cytosolic ascorbate peroxidase (Karpinski et al. 1999). The peroxisome is likely to be a factor of importance in the hydrogen peroxide-dependent redox regulation since it is a major source of photorespiratory peroxide.

The mechanisms that regulate the biogenesis and capacity of the cyt path in light are not clearly understood. However, it is assumed that in light, as compared to darkness, the phosphorylating cyt path is relatively less active than the non-phosphorylating alternative respiratory pathway (Rasmusson and Svensson, 2001). The data available suggest that the accumulation levels of the proteins involved in the oxidative phosphorylation are unaffected by light (Dudley et al. 1997; Hedtke et al. 1999; Rasmusson and Svensson, 2001). In addition, in developing pea leaves, cyt path activity was found unchanged while the GDC and the AOX increased activity, the latter mainly via the increased reduction of the AOX enzyme (Lennon et al. 1995). Furthermore, when the capacity of the cyt path respiration is altered (using for example, antimycin A or cysteine), a coordinated increase in the AOX protein level is observed while the levels of proteins of the cyt path remain stable (Vanlerberghe and McIntosh 1997; Vanlerberghe et al. 2002). The down-regulation of the cyt path capacity in cysteine-treated tobacco cells seems to involve a process of dephosphorylation (Vanlerberghe et al. 2002), which might occur via a two component redox regulatory system. The regulation of the cyt path capacity/activity is also likely to be explained by a regulation of the partitioning of the electrons between the cyt path and the alternative pathway at the UQ pool; in soybean cotyledons, pyruvate increases electron partitioning through the alternative pathway under both state 3 and state 4 (Ribas-Carbo et al. 1995; 1997). Electron partitioning may be influenced by the cytosolic ATP/ADP ratio. During photosynthesis, the demand for mitochondrial ATP comes for an important part from sucrose synthesis and N-assimilation (Raghavendra et al. 1994; Gardeström and Lenmark 1995; Krömer 1995, Hoefnagel et al. 1998), and depends therefore on the photosynthetic conditions such as light intensity and the availability of carbon dioxide and nutrients. Mitochondria provide most of the cytosolic ATP for sucrose synthesis. In conditions of high ATP/ADP ratios or limiting ADP (during high photorespiration for example), the proton gradient dissipation across the inner mitochondrial membrane is lowered, leading to the increase of the cyt path redox state. This can cause, besides the activation of uncoupler protein (UCP) (Sluse and Jarmuszkiewicz 2000), a further engagement of the alternative pathway in order to regulate the redox state and avoid too high levels of reducing equivalents (Day and Wiskich 1995; Vanlerberghe and McIntosh, 1997). The tendency observed is clearly towards the activation and expression of components of the non-phosphorylating pathways in the light and vice-versa in the dark whereas components of the cyt path remain in place with mainly its activity controlled. This may be related to the necessity of quick adaption to the energy demands, that can arise from different environmental conditions. The proteins of the alternative pathway are relatively small and can be synthesized quickly, in

contrast to the respiratory complexes, which require the assembly of multiple subunits into complexes ranging from 150 to 800 kDa.

In *C. elongatum*, an increased expression has been observed of cytochrome *b* (*cob*) in the dark (Kroymann et al. 1995). It is possible that for unicellular green algae, the light-regulation occurs at a different level since every cell is in direct contact with its physical environment (growth medium), necessitating a different level of regulation than in plants. Also, frequently the culture media used for the growth of alga uses acetate or other carbon sources, which could give rise to different responses.

Finally, the accumulation of proline can be caused by many different stress conditions, including photo-oxidative stress. The interconversion of proline, Delta-pyrroline-5-carboxylate (P5C) and glutamate was proposed to constitute a form of cellular redox signaling (Hare and Cress 1997). In the mitochondrion, the conversion of proline to P5C by proline oxidase yields $FADH_2$ whereas NADH is generated by the conversion of P5C into glutamate, which can then be converted into the TCA-cycle intermediate 2-oxoglutarate. When imported into the mitochondrion, proline could constitute a photosynthetic redox messenger, provided that the stress conditions that cause the proline accumulation do not inhibit mitochondrial function as well.

Concluding remarks

The presence of two major bioenergetic processes in the photosynthetic cells implies a tight and complex regulation of plant organelle biogenesis and activity. In particular, a concerted expression of the three genomes in a green cell is essential to adapt to the various environmental conditions. The modulation of function and gene expression by light or with leaf development and the obvious link between light and cellular redox state or redox-transducing messages, suggest that the redox state has an important regulatory role not only in the chloroplast, but in the whole cell and even in whole tissues and plants. For example, the cytoplasmic thioredoxin *h* was proposed to act as a redox messenger between different plant tissues via the phloem of for example rice and wheat (Schürmann and Jacquot 2000). It is imaginable that oxygen plays a role in cellular light regulation as well. For instance, the up-regulation of glycolysis by the induction of hexokinase under low oxygen pressure (Koch et al. 2000) may constitute a factor in the regulation of mitochondrial respiration in the dark. Also, the role of photoreceptors such as phytochrome in the redox regulation is beginning to be unraveled (Neuhaus et al. 1997), but is known already for a long time to regulate mitochondrial components such as the cytochrome *c* oxidase (Hilton and Owen 1985).

Further 'cross-talk' studies will increase our understanding of the extent to which redox regulation is important for the adaptation of mitochondrial function and biosynthesis. The pursuit of the role of photosynthetic redox messages, of which some have been mentioned in this review, will likely prove to be a substantial contribution to our understanding of how mitochondria 'feel' the light stimulus.

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Legend

Figure 1

Schematic representation of various chloroplast-originated redox messages that are potentially involved in the signal transduction of the light regulation of mitochondrial function and biogenesis. Light is a factor of decisive importance, besides other factors as carbon dioxide and nutrients, in the generation and export of redox-related metabolites and messages. These can be transmitted to the mitochondrion and effectuate adaptive responses through the effect on the mitochondrial redox state, notably the NADH/NAD ratio and the reduction state of the respiratory chain. Redox messages can also be relayed to the nucleus and affect expression of genes for mitochondrial proteins. Redox-dependent light regulation of nuclear gene expression include two component or similar redox regulation, or involve hydrogen peroxide-induced signals. Hydrogen peroxide influences ratios of oxidized versus reduced antioxidants such as glutathione and ascorbate, which on its turn may modulate gene expression.
Chloroplast and mitochondrial translocaters or shuttle systems are indicated by a circle. Enzymes (systems) are in small font. Phospho protein refers to light- and redox-induced protein phosphorylation. Mit. proteins are mitochondrial proteins. e^- represents the reduction level of the ETC.
Acet-CoA, acetyl coenzyme A; ATP/ADP, adenlyate status; DHA/ASC, ratio of dihydroxy ascorbate and ascorbate; GSSH/GSH, ratio of glutathione disulphide and reduced glutathione; NADH/NAD, ratio of reduced versus oxidized pyridine nucleotides; DHAP, dihydroxyacetone phosphate; ETC, electron transport chain; OAA, oxaloacetic acid; PEP, phophoenol pyruvate; PGA, 3-phophoglycerate; PQ, plastoquinone pool; RuBP, ribulose 1,5-biphosphate; TCRR, two component or similar redox regulation; UQ, ubiquinone pool.
Enzymes: AOX, alternative oxidase; CS, citrate synthase; GDC, glycine decarboxylase complex; GO, glycolate oxidase; GP, glyceraldehyde 3-P dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; MR, Mehler reaction; PDC, pyruvate decarboxylase complex; Rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase.

APÉNDICE IV

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6 Transfer of mitochondrial genes to the nucleus in chlamydomonad algae: Perspectives for the allotopic expression of OX-PHOS proteins and future human therapies

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Abstract

Mitochondrial genomes encode a limited set of polypeptides that are components of the membrane-embedded oligomeric complexes that participate in oxidative phosphorylation (OX-PHOS). It has been proposed that the genes that remained localized in the mitochondrial genome are those that encode highly-

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hydrophobic polytopic proteins, containing two or more helices that span the membrane. The 15.8 kb linear mitochondrial genome (mtDNA) of the green alga Chlamydomonas reinhardtii lacks several of the genes, encoding essential components of OX-PHOS, that are typically found on mtDNA, including cox2, cox3 and atp6. These genes were transferred to the nucleus of this alga where they were modified to permit their successful expression in the nucleus of C. reinhardtii. In an extreme example, the cox2 gene, encoding the COXII subunit, has been split into two genes whose products, COX IIA and COX IIB are predicted to form a heterodimeric subunit. The predicted COX IIA, COX III, and ATP6 polypeptides have long putative mitochondrial targeting sequences (MTS) and exhibit diminished physicochemical constraints for import into mitochondria. The mean hydrophobicity of the nucleus-encoded proteins is diminished, particularly in those transmembrane stretches that are thought not to participate directly in proton translocation or in inter-subunit contacts, as shown for the ATP6 and COXIII subunits. Based on these results, we suggest some conclusions on the allotopic expression of human mitochondrial genes and on the design of mitochondrial gene therapy strategies.

1. Introduction

In some members of the chlorophyte algae from the family Chlamydomonadaceae, several genes that are normally found in the mitochondrial genomes, like *cox2*, *cox3*, and *atp6*, naturally reside in the nucleus. The study of these genes, and the structural changes that allowed their successful relocation to the nucleus, are the subject of this review. To illustrate this transfer process, we first briefly address the large diversity of mitochondrial genomes in nature, and in particular, those of Chlorophycean algae. We then summarize the evidence for the prevalent and on-going transfer of mitochondrial genes to the nucleus, review this process in chlamydomonad algae, and address why some genes have remained in mitochondrial genomes. We suggest that nuclear genes encode mitochondrial membrane proteins whose overall hydrophobicity has decreased as compared to its mitochondria-encoded counterparts. We also show that the hydrophobicity has decreased preferentially in those transmembrane regions of the protein that seem not to be critical for function or for inter-subunit interactions. Finally, we suggest some conclusions for consideration when designing mitochondrial gene therapy strategies involving allotopic expression of mitochondrial genes.

2. The large diversity of mitochondrial genomes in nature

Mitochondria are believed to have evolved from endosymbionts [1,2] that derived originally from free-living α-proteobacteria [3,4,5,6], related to the

contemporary members of the genus *Rickettsia* [7]. The nature of the host with whom the endosymbiosis was established remains a matter of debate, and it is thought to be either an archeon or an amitochondrial eukaryote [8]. It is believed that the endosymbiotic event that gave rise to mitochondria occurred only once in evolution [9], and that it was followed by a massive transfer of genes to the nucleus, followed by a more gradual transfer [10] that seems to be an ongoing process [11]. This long-term migration process gave rise to the present highly reduced mtDNAs.

Mitochondrial genomes from different species vary in size as well as in organization. In general, they encode ribosomal RNAs, tRNAs, and a limited set of polypeptide subunits of OX-PHOS proton translocating complexes, plus a variable set of proteins involved in mitochondrial protein synthesis [5]. The majority of mitochondrial proteins are nucleus-encoded, translated in the cytoplasm, and translocated through a specialized import-machinery known as the TOM-TIM complex [12]. In yeast, more than 400 mitochondrial proteins (97% of all proteins required for mitochondrial function) are encoded in the nucleus [13]. Many proteins imported into mitochondria contain a mitochondrial targeting sequence (MTS), generally a small, cleavable presequence of 20 to 40 amino acids, capable of forming an amphiphilic α-helix, that is recognized by the mitochondrial TOM-TIM machinery.

A wide spectrum of gene content of mitochondrial genomes is found in nature. The 60 kb mtDNA of the flagellate *Reclinomonas americana* [14], *"the mitochondrion that time forgot"* [15], resembles a highly reduced bacterial genome. This mtDNA encodes 24 proteins that participate in OX-PHOS, plus a set of 38 additional proteins involved in translation, transcription, protein import, and maturation. The *R. americana* mitochondrial genome is thought to be the extant mtDNA that most closely resembles the proto-mitochondrial genome. At the other end of the mtDNA complexity-spectrum, there is the highly reduced 6 kb mtDNA of the apicomplexan parasite *Plasmodium falciparum*, that contains only 3 genes encoding components of the mitochondrial respiratory chain - *cob*, *cox1*, and *cox3* [16,17]. *cob* and *cox1* are present in all mitochondrial genomes known to date. Their gene products are highly hydrophobic polytopic proteins that function as central components of proton translocating complexes: cytochrome *b* of the bc_1 complex, and subunit I of cytochrome *c* oxidase. In the small circular mitochondrial genomes of metazoans, including humans, 13 protein-coding genes, all encoding components of oxidative phosphorylation are present: *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *cob*, *cox1*, *cox2*, *cox3*, *atp6*, *atp8* [18].

3. The mitochondrial genomes of chlorophycean algae

The class Chlorophyceae, members of the phylum Chlorophyta (green algae), contains more than 355 genera and 2650 species. Most live in

freshwater but there are also marine and terrestrial species [19]. The mitochondrial genomes of 7 species of chlorophycean algae have been sequenced, *Chlamydomonas eugametos* [20], *Scenedesmus obliquus* [21,22], *Chlorogonium elongatum* [23], *Chlamydomonas reinhardtii* [24,25], *Nephroselmis olivacea* [26], *Pedinomonas minor* [26], and *Prototheca wickerhamii* [27]. Table 1 summarizes the gene content of the mitochondrial genomes of these green algae. The 15.8 kb linear mtDNA from *C. reinhardtii*

Table 1. Gene content in the mitochondrial genomes of Chlorophycean algae.

Genes	TR	*P. wi*	*N. ol*	*P. mi*	*S. ob*	*C. re*	*C. eu*	*C. el*
COMPLEX I								
nad1	8-9	+	+	+	+	+	+	+
nad2	13-14	+	+	+	+	+	+	+
nad3	3	+	+	+	+	-	-	-
nad4	13-14	+	+	+	+	+	+	+
nad4L	3	+	+	+	+	-	-	-
nad5	15-16	+	+	+	+	+	+	+
nad6	5	+	+	+	+	+	+	+
nad7	1-2	+	+	-	-	-	-	-
nad8	1	-	-	-	-	-	-	-
nad9	1	+	+	-	-	-	-	-
COMPLEX III								
cob	9	+	+	+	+	+	-	+
COMPLEX IV								
cox1	12	+	+	+	+	+	+	+
cox2	2	+	+	-	+ (*cox2a*)	-	-	-
cox3	7	+	+	-	+	-	-	-
COMPLEX V								
atp1	0	+	+	-	-	-	-	-
atp6	5	+	+	+	+	-	-	-
atp8	2	+	+	+	-	-	-	-
atp9	2	+	+	-	+	-	-	-
rib RNAs								
rnl		+	+	+ (2)	+ (4)	+ (8)	+ (6)	+ (6)
rns		+	+	+	+ (2)	+ (4)	+ (3)	+ (3)
5S		+	+	-	-	-	-	-
tRNAs		26	26	8	27	3	3	3

TR= transmembrane regions of the protein encoded, *P. wi= Prototheca wickerhamii, N. ol=Nephroselmis olivacea, P. mi= Pedinomonas minor, S. Ob= Scenedesmus obliquus, C. re= Chlamydomonas reinhardtii, C. eu= Chlamydomonas eugametos, C. el= Chlorogonium elongatum.* Numbers in parenthesis indicate the number of fragments that constitute the ribosomal RNAs

lacks several genes encoding essential components of OX-PHOS: *nad3*, *nad4L*, *cox2*, *cox3*, *atp6*, and *atp8*, that until recently had only been characterized as mtDNA-encoded genes. These six genes are also absent from the circular genomes of the related green algae *C. eugametos* and *C. elongatum*. It was hypothesized that these genes had been transferred to the nucleus in these species. The products of two of these genes, subunit III of cytochrome *c* oxidase (COX III) and subunit 6 of F_1F_0-ATP synthase (ATP6, equivalent to subunit *a* in *Escherichia coli*), are usually highly-hydrophobic polypeptides, with 7 [28] and 5 [29,30] transmembrane stretches respectively. As outlined below, we have demonstrated that functional *atp6*, *cox2*, and *cox3* genes are nuclear localized in *C. reinhardti* [31,32,33]. The modifications that enabled these once mitochondrial genes to become nuclear localized and fully functional offers insights into the evolutionary processes involved, and suggest possible strategies for the allotopic expression of proteins as potential genetic therapies for human mitochondrial disorders.

4. Transfer of mitochondrial genes to the nucleus

The theory of mitochondrial origin proposes that there was a gradual transfer of genes from the endosymbiont to the nucleus [10]. This transfer of genetic material may have happened in the form of DNA or of cDNA [34]. In *S. cerevisiae*, the current rate of transfer of DNA from the mitochondria to the nucleus is 10^5 times more frequent than the rate of transfer in the opposite direction [35]. This suggests a favored unidirectional flux of genetic material from organelles to nuclear control. Transfers of mtDNA to the nucleus can involve fragments ranging from 31 nt [36] up to 620 kb, as in the case of *Arabidopsis thaliana*, where a complete mtDNA copy was found in the nucleus [37]. The human genome project has also revealed transfer of large mitochondrial fragments to the nucleus, ranging from 106 to 14,654 bp [38].

The continous transfer of organellar DNA to the nucleus predicts that all coding sequences should eventually be displaced from the mitochondrion [39]. However, this transfer is not always successful, since on many occasions genes do not establish themselves functionally in the nucleus. Numerous copies of mitochondrial genes reside in the nucleus as pseudogenes in over 64 animal species [40,41]. These pseudogenes are considered relics of anciently transferred mtDNA that remain as molecular fossils in the nucleus [42]. Mitochondrial DNA has been transferred to the nucleus on numerous independent occasions, and the same gene can be sent to the nucleus at different times in closely related organisms [9]. These multiple transfers sometimes resulted in a successful integration of the genes and their establishment as functional genes. In other occasions, after integration, the genes were not activated, and remained as pseudogenes.

Transfer of mitochondrial genes to the nucleus seems to be an ongoing process [43], exemplified by the presence of similar genes for the same protein encoded in both the mitochondrial and the nuclear genomes of an organism, i.e. F_1F_0-ATP synthase subunit 9 (ATP9) of *Neurospora crassa* [44,45] and cytochrome c oxidase subunit II (COX II) of some higher plants [11,46]. The functional relocation of mitochondrial genes to the nucleus required several steps [39,47]: *i)* The transfer of a gene to the nucleus, while an active copy is still retained in the mitochondria. *ii)* The activation of the nuclear copy of the gene by acquisition of sequences encoding nuclear promoters, ribosome binding sites, mitochondrial targeting sequences (MTS), and polyadenylation signals. These structural transformations may also be accompanied by changes in codon usage and the acquisition of introns. The relocalization of mitochondrial genes to the nucleus implies the coexistence of active nuclear and mitochondrial genes during a variable period of time, as described for the *cox2* gene in some legumes [48]. *iii)* Inactivation of the mitochondrial gene. Such is the case of the mitochondrial ribosomal protein S14 in rice, that has been transferred to the nucleus, while the original mitochondrial copy has been interrupted by stop codons [49]. Ultimately, the original mitochondrial gene will be eliminated. Alternatively, the nuclear copy may be the one to inactivate, resulting in the original gene being retained in the mitochondrion and the appearance of mtDNA pseudogenes in the nucleus [50].

The transfer of mitochondrial genes to the nucleus may confer a selective advantage, since nuclear genes in some organisms exhibit a lower mutation rate [51]. This is not necessarily true of fungi, where mutation rates of the nuclear and mitochondrial genes are almost equivalent, or in plants, where nuclear genes mutate at a higher rate than mitochondrial genes [52,53,54]. Gene transfer from organelles to the nucleus is also thought to reduce the accumulation of deleterious mutations, since the genes migrate from a predominantly asexual to a predominantly sexual genome [55,56].

5. Transfer of mitochondrial genes to the nucleus in chlamydomonad algae

Since the genes *cox2*, *cox3*, and *atp6* were absent in the mtDNA of *C. reinhardtii*, we investigated whether these genes were expressed in the nucleus. We used the standard techniques of molecular biology to address the presence of these genes in *C. reinhardtii* combined with a biochemical approach to show the presence of the corresponding proteins in the mitochondrion. For this second approach, we also used the colorless alga *Polytomella* sp. as it lacked a cell wall and functional chloroplasts [57,58]. These characteristics allowed the isolation of algal mitochondria free of thylakoid contaminants which facilitated the purification of several OX-PHOS complexes [31,59,60,61]. The colorless algae

of the genus *Polytomella* are closely related to *C. reinhardtii*, as demonstrated by the similarities between their nucleus-encoded beta-tubulin [62] and 18S rRNA [58,63], and mtDNA-encoded *cox1* genes [64].

The combined studies with *C. reinhardtii* and *Polytomella* sp. demonstrated that the gene *cox3*, encoding subunit III of cytochrome *c* oxidase (COX III), had been transferred to the nucleus in both organisms [31]. We also found that in both *Polytomella* sp. and *C. reinhard*tii subunit II of cytochrome *c* oxidase (COX II) is encoded by two separate nuclear genes that were named *cox2a* and *cox2b*. The *cox2a* gene encodes a protein, COX IIA, corresponding to the amino terminal half of a typical single-polypeptide COX II, that includes the two transmembrane stretches. The *cox2b* gene encodes a protein, COX IIB, equivalent to the soluble C-terminal domain of an orthodox COX II subunit. We proposed that the separate *cox2a* and *cox2b* genes gave rise to a heterodimeric COX II that resulted from the non-covalent assembly of the COX IIA and COX IIB polypeptides in the mature cytochrome *c* oxidase complex [32]. This contrasts with the COX II proteins of other eukaryotes, that are single polypeptides encoded by single genes normally localized in the mitochondrial genome, with the exception of full-length nuclear *cox2* genes in the nuclei of some legumes. In addition, the gene *atp6* encoding subunit ATP6, an essential component of the proton translocating F_0 sector of the F_1F_0-ATP synthase, has also been transferred to the nucleus in *C. reinhardtii* [33]. Therefore, the four genes *cox2a, cox2b, cox3*, and *atp6* where shown to reside in the nucleus in at least some members of the family Chlamydomonadaceae, in contrast to the mitochondrial location of the genes *cox2, cox3* and *atp6* in the vast majority of eukaryotes. The transfer of these genes probably occurred late in evolution, after the massive transfer of genes from the protomitochondrion to the nucleus [5], since nowadays many green algae still retain the *cox2, cox3*, and *atp6* genes in their mitochondrial genomes.

The discovery of these genes in *C. reinhardtii* and *Polytomella* sp. allows a consideration of the specific features that accompanied their transfer from the mitochondrion to the nucleus:

a) **The export of the nucleic acid molecule from the mitochondria in the form of DNA or RNA.** Some genetic material has been transferred to the nucleus via RNA intermediates, shown by the presence of edited versions of plant mitochondrial genes in the nucleus, where the mRNA has a change of C→U when compared to the genomic sequence. [46,48,65,66]. This transfer as RNA must have been followed by a reverse transcription step, before its integration into the nuclear genome. It remains to be ascertained if the genes *atp6, cox2a, cox2b*, and *cox3* of chlamydomonads were transferred as DNA or RNA intermediates. Mitochondrial editing has not been found in algae, and no evidence for reverse transcription activity has been found,

despite the presence of a gene encoding a putative reverse transcriptase-like protein in the mitochondrial genome of *C. reinhardtii* [24]. The absence of editing suggests that the integration of an RNA-derived DNA fragment was not obligatory, as would be the case in an organism that required RNA editing for functional gene expression.

b) **Integration into the nucleus.** Integration into the nuclear genome most probably occurred in a non-coding region through nonhomologous recombination [67], or by an end-joining mechanism [36].

c) **Acquisition of an MTS.** Random genomic DNA sequences preceding mitochondrial genes that have been relocalized in the nucleus could provide an MTS. Two to five % of randomly sheared DNA sequences attached upstream of protein genes *in vitro* functioned as effective MTS [68], showing the potential ease of gaining of an MTS. In some cases, mitochondrial genes have inserted into nuclear genes, acquiring the pre-existing MTS [48,69]. MTS acquisition may also occur by duplication of existing targeting signals [70] or by nuclear exon shuffling [71]. The deduced amino acid sequence located before the N-terminal sequence of the mature COX IIA, COX III and ATP6 proteins of *C. reinhardtii* predicts the existence of unusually large MTSs, of 143, 119 and 107 amino acids respectively, rich in alanines, prolines, and charged amino acids. In yeast, it has been observed that the duplication of MTSs improves the *in vitro* and *in vivo* import of hydrophobic proteins into mitochondria [72]. It has also been suggested that long MTSs can improve the interaction of the precursor with the mitochondrial import machinery [73]. Alternatively, a long MTS could affect the folding of the protein to increase its importability [74]. It is possible that, during import into mitochondria, the targeting sequences are cleaved and maintained as components of the cytochrome *c* oxidase complex, as was observed with the MTSs of the Rieske subunit of yeast and beef heart mitochondrial cytochrome bc_1 complexes [75,76]. The function of the targeting sequence retained in the bc_1 complex is not known, we speculate that it may act as a chaperone for components of the enzyme complex during assembly. A conventional N-terminal MTS is not absolutely required for a nucleus-encoded protein to be imported into the mitochondrion. Several mitochondrial genes that were transferred to the nucleus have become activated, and their protein products may be imported into mitochondria in the absence of an MTS [50]. This is also the case for the *cox2b* genes of *C. reinhardtii* and *Polytomella* sp. which do not exhibit a region encoding a putative MTS.

d) **Acquisition of introns, promoters, and ribosome binding sites.** Most nuclear genes in *C. reinhardtii* have introns, with an average number of 3.9 introns per kb of coding sequence. These introns are often small, ranging from 57 bp to 1318 bp, with an average size of 219 bp [77]. We found

introns in the genomic sequences of the chlamydomonad mitochondrial genes that were transferred to the nucleus: 6 in *cox2a* of *Polytomella* sp., 7 in *cox2a* of *C. reinhardtii*, 1 in *cox2b* of *C. reinhardtii*, 4 in *cox3* of *Polytomella* sp., 9 in *cox3* of *C. reinhardtii*, and 7 in *atp6* of *C. reinhardtii*. These introns show orthodox splicing sites exhibiting the typical GT sequence at the 5' end and an AG sequence at the 3' end. An exception was *Polytomella* sp. *cox2b* that did not contain introns. The sequences flanking the proposed open reading frame initiating methionine codons corresponded to the consensus translation initiation site (A/C) A (A/C) (A/C) ATG (G/C) reported for *C. reinhardtii* [77]. We have yet to identify promoter regions in the genomic regions upstream from these genes.

e) **Acquisition of polyadenylation signals.** In *C. reinhardtii* the most common polyadenylation signal in the nuclear genes is TGTAA located 10-20 bp upstream of the actual polyadenylation site [77]. This signal was present in the *cox3*, *cox2a*, *cox2b*, and *atp6* genes that were transferred to the nucleus in chlamydomonad algae. These signals are distinctive features of nuclear genes, since they are not present in mitochondrial genes.

f) **Change in codon usage.** The *C. reinhardtii* mitochondrial genetic code is the same as the standard genetic code utilized in the nucleus, which may facilitate a continued transfer of mitochondrial genes to the nucleus. However, the chlamydomonad algae exhibit a highly biased codon usage in their nuclear genes. The nuclear genome of *C. reinhardtii* has a high GC content, and this feature is reflected in the pronounced codon bias, which favors triplets with C or G in the third position. In contrast, there is a different bias in the codon usage in the mitochondrial genome. Relocalization of mitochondrial genes to the nucleus in *C. reinhardtii* was followed by changes in codon usage to frequencies typically found for nuclear genes.

g) **Inactivation of the mitochondrial gene copy, and loss of the original mitochondrial gene.** Successful transfer of genes from organelles to the nucleus is usually followed by inactivation of the mitochondrial copy, its conversion into a pseudogene, and its eventual loss from the mitochondrial genome [78]. There is no evidence for the presence of *cox2a*, *cox2b*, *cox3* or *atp6* genes, gene fragments, or pseudogenes in the mitochondrial genomes of several chlamydomonads, including *Polytomella* sp., *C. reinhardtii*, *C. eugametos*, *C. elongatum* and *C. moewusii*.

h) **The splitting of the cox2 gene into cox2a and cox2b.** The gene encoding the mitochondrial COX II protein is absent from the mtDNA of a number of chlamydomonad algae. We found that the gene had been split into two parts, *cox2a* and *cox2b*, both expressed from the nucleus, in *Polytomella* sp. and *C. reinhardtii* [32]. We also observed that a *cox2* gene in the mtDNA of *Scenedesmus obliquus* shows strong similarities to the chlamydomonad

cox2a gene, suggesting that the splitting of *cox2* occurred prior to the transfer of the gene to the nucleus, and that the chlamydomonad transfer of *cox2b* to the nucleus is likely to have preceded that of *cox2a*. It is not known if the *S. obliquus* mtDNA-encoded *cox2a* is functional, or if there is also a nuclear copy of the same gene. It is possible that the *S. obliquus cox2a* was prevented from relocating to the nucleus by the divergence of the mitochondrial genetic code from the standard code in this organism [21,22]. In *Polytomella* sp. and *C. reinhardtii* the COX IIA protein contains a C-terminal 20-amino acid region, lacking similarity to conventional COX II proteins, that had a high density of charged amino acids. The predicted COX IIB polypeptide contains 42-amino acids at the N-terminus with a high density of charged amino acids that are not homologous to known COX II proteins and are not a cleavable MTS. We propose that the C-terminal extension of COX IIA interacts with the N-terminal extension of the COX IIB protein and that these acquired amino acid sequences stabilize the two COX II subunits in the cytochrome *c* oxidase complexes.

6. Why have some genes remained in the mtDNA?

Several ideas have been put forward to explain why mitochondrial genomes still contain a limited set of genes that has not been transferred to the nucleus. One explanation has been the evolution of a different mitochondrial genetic code in some organisms that would inhibit the functional expression of mitochondrial genes transferred to the nucleus. Similarly, some mitochondrial genes have accumulated complex processing patterns like mRNA-editing, which would render the transferred gene inviable when relocated to the nucleus [79]. Another explanation may be that the presence of some organellar proteins in the cytoplasm could have detrimental effects [80], including misrouting of certain highly hydrophobic mitochondrial proteins synthesized in the cytosol to other cell structures, such as the endoplasmic reticulum [81]. An additional explanation suggests that some genes have remained in the mitochondrial genome to be rapidly regulated by the organelle redox state [82]. Finally, it has been proposed that highly hydrophobic proteins, containing four or more helices that span the membrane, can not be readily imported into mitochondria. Therefore, these polytopic membrane proteins must be synthesized *in situ* to be properly inserted and assembled into the inner mitochondrial membrane [74,83]. The synthesis of these hydrophobic polypeptides inside the mitochondria, may ensure their proper insertion in the inner membrane, giving rise to the correct topological arrangement required for vectorial proton translocation.

There are two universal examples of genes retained in the mitochondrial genomes: the cytochrome *b* gene (*cob*), encoding a protein with 8 transmembrane regions [84], and the cytochrome *c* oxidase subunit I (*cox1*),

which encodes a protein with 12 transmembrane helices [28,76]. These subunits also bind prosthetic groups, including heme groups and metal ions. Both genes are present in all mitochondrial genomes so far characterized. Other genes that encode highly hydrophobic polypeptides (*cox2, cox3, atp6, atp8, atp9, nad1, nad2, nad3, nad4, nad4L, nad5* and *nad6*) are also present in the majority of mtDNAs [85]. In some yeasts, the absence of *nad* genes in the mitochondrial genome is related to the lack of respiratory complex I. Those organisms that do contain complex I retain a set of six to seven *nad* genes. The corresponding proteins of the genes retained in mtDNAs exhibit at least two transmembrane helices, and some of them up to 17 putative transmembrane stretches. Therefore, it may be that physicochemical properties (mainly hydrophobicity) are the ultimate limiting step for the transfer of mitochondrial genes, encoding polytopic membrane proteins, to the nucleus.

7. Nuclear genes encoding mitochondrial membrane proteins decrease their hydrophobicity when compared to their mitochondrial counterparts

In yeast, *in vivo* studies with cytoplasmic synthesized constructs of variable lengths of apocytochrome *b*, showed that the importability of polypeptides into mitochondria is not strictly related to the number of transmembrane domains [74]. These studies suggested that the highest average hydrophobicity over 60 to 80 amino acids of a polypeptide chain (*meso*H), along with the maximum hydrophobicity of the putative transmembrane segments, are useful indicators of the likelihood that a protein could be imported into mitochondria. Accordingly, mitochondrial *cox2, cox3* and *atp6* genes from many eucaryotes encode proteins that exhibit physicochemical characteristics that would block the import of such proteins if they were nucleus-encoded. However, the corresponding nucleus-encoded proteins of chlamydomonad algae COX IIA, COX IIB, COX III and ATP6, all exhibit reduced overall hydrophobicity that allow them to be imported into mitochondria, most probably through the TOM-TIM machinery. Figure 1 shows a mesohydrophobicity (*meso*H) versus maximal local hydrophobicity (<H>) plot for the *C. reinhardtii* and *Polytomella* sp. COX IIA, COX IIB, COX III and ATP6 sequences as compared to the protein products predicted from the human mitochondrial genes nad1, *nad2, nad3, nad4, nad4L, nad5, nad6, cob, cox1, cox2, cox3,* and *atp6*. The *atp8* gene was not included since its very small size, 68 amino acids, distorts the *meso*H and <H> values. In comparison with their human mtDNA-encoded counterparts, the nucleus-encoded chlamydomonad sequences display both decreased *meso*H and <H> (Figure 1). We propose that one of the important modifications required for nuclear genes to encode functional mitochondrial OX-PHOS proteins is a diminished overall

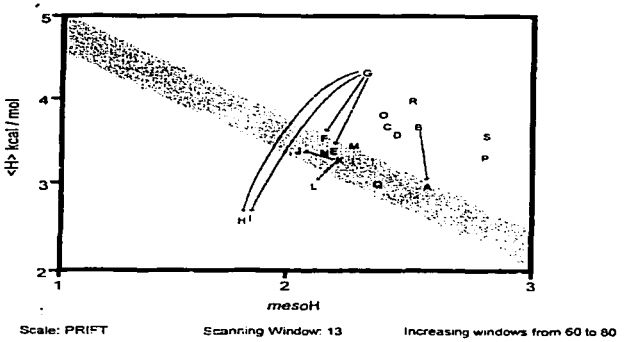

Figure 1. Plot of mesohydrophobicity (*meso*H) versus maximal local hydrophobicity (<H>) of the OX-PHOS mitochondrial proteins encoded in the human mtDNA compared to the nucleus-encoded homologs COX II, COX III and ATP6 of *Chlamydomonas* and *Polytomella*. Arrows indicate the mitochondrial and nuclear counterparts. Letters indicate the following sequences: A. *C.reinhardtii* ATP6, B. human ATP6, C. human COB (cytochrome *b*), D. human COX I, E. *C. reinhardtii* COX IIA, F. *Polytomella* sp. COX IIA, G. human COX II, H. *C. reinhardtii* COX IIB, I. *Polytomella* sp. COX IIB, J. *C. reinhardtii* COX III, K. human COX III, L. *Polytomella* sp COX III., M. human NAD1, N. human NAD2, O. human NAD3, P. human NAD4L, Q. human NAD4, R. human NAD5, S. human NAD6.

mesohydrophobicity of its protein product. The changes in hydrophobicity may have occurred either prior or subsequently to the transfer process and may allow for the successful import and assembly of these proteins into the mitochondrial inner membrane.

Mitochondria readily import hydrophobic carrier proteins with multiple transmembrane stretches, like the adenine nucleotide translocator. Nevertheless, carrier proteins also seem to follow the low <H> and low *meso*H rule [74]. However, the import pathway of the translocators differs greatly from the "conservative intramitochondrial sorting pathway", in which polypeptides are transferred to the mitochondrial matrix space and then sorted to its final membrane destination. The insertion of carrier proteins into the mitochondrial

inner membrane is mediated through different TIM complexes, mainly the so-called tiny-TIMS and the TIM22 complex [12]. This import pathway is distinct from the one followed by orthodox MTS-containing polypeptides.

8. Mean hydrophobicity has decreased in those transmembrane regions that are not critical for function

Hydropathy analysis of the COX III polypeptides of *Polytomella* sp. and *C. reinhardtii* showed the presence of seven putative transmembrane stretches, numbered I to VII. The hydrophobicity of these seven helices seems to be lower in the chlamydomonad algae when compared with the *Paracoccus denitrificans* or the bovine subunits. This was more evident when the three dimensional structure of COX III from *Polytomella* sp. was modelled using the crystallographic coordinates of the bovine subunit [28]. In this model (Figure 2), shorter transmembrane stretches are observed as well as interruptions in the middle-sections of the membrane helices. The helices that are in contact with COX I (helices I and III), do not exhibit significant modifications in hydrophobicity. However, transmembrane domains II, V and VI have diminished their hydrophobicity by 16%, 10% and 12%, respectively. It should be noted that domains IV and VII, that are the least hydrophobic in the mtDNA-encoded COX III proteins (ΔG ~1.1 kcal/mol) offer distinct behaviour: IV remains the same and VII has increased its hydrophobicity by 22%, although the final hydrophobicity is not predicted to be a problem for import. This suggests that the diminished hydrophobicity of COX III is stronger in those regions of the protein which seem not to be involved in subunit-subunit interactions (Figure 3).

The *in silico* analysis of the predicted ATP6 sequence of *C. reinhardtii* revealed similar characteristics [33]. Based on multiple sequence comparisons, secondary structure predictions, and available biochemical data [29,30, and see also 118], five hydrophobic regions could be predicted as transmembrane stretches and were named A, B, C, D and E. Hydrophobicity analysis showed that transmembrane helices A, B and C exhibit a highly reduced mean hydrophobicity. In fact, helix A could even be considered not to be membrane-imbedded. A similar situation was observed for the transfer to the nucleus of the mitochondrial *sdh3* gene - encoding subunit 3 of succinate dehydrogenase - in angiosperms: its protein product is predicted to lack one of three transmembrane domains [86]. In contrast ATP6 transmembrane helices D and E, which are believed to interact with the multimeric ring of *c*-subunits (ATP9), and which contain most of the conserved amino acids in the protein, exhibit similar <H> values when compared with the helices of other mitochondria-encoded ATP6 subunits.

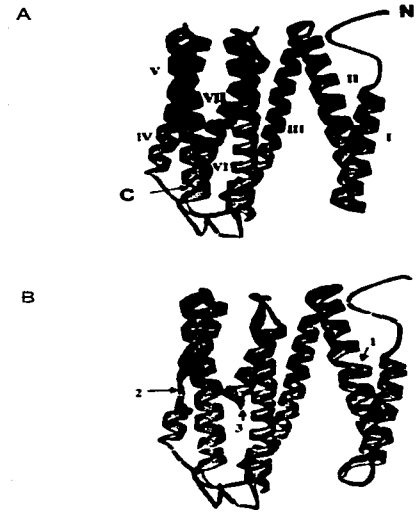

Figure 2. Model for the structural arrangement of COX III from *Polytomella* sp. **Panel A**, three dimensional structure of bovine COX III [28]. The seven transmembrane helices are indicated by roman numerals. The amino and carboxy termini are indicated by the letters N and C, respectively. **Panel B**, three dimensional model for Ps-COX III based on the bovine structure. Arrows indicate distinct features of this polypeptide: 1) the presence of a kink in transmembrane helix II, 2) a distortion in the middle-section of transmembrane helix IV, and 3) a shorter transmembrane helix VII.

Transmembrane domains II in COX III and helix A in ATP6 are the most hydrophobic, and have decreased their <H> to the greatest extent. Therefore, we conclude that the overall decrease in hydrophobicity in the chlamydomonad subunits COX IIA, COX III, and ATP6 accompanied the functional transfer of their respective genes to the nucleus, and facilitates the import and assembly of

these proteins into active cytochrome *c* oxidase and F_1F_0-ATP synthase complexes, respectively. In addition, we propose that the selective decrease in the hydrophobicity of proteins encoded by nuclear genomes is stronger in those transmembrane regions that seem not to be critical for function, assembly, or participation in inter-subunit interactions with other constituents of the complexes. This conclusion has important implications for the allotopic expression of mitochondrial genes and for future application of allotopic expression to human mitochondrial gene therapy.

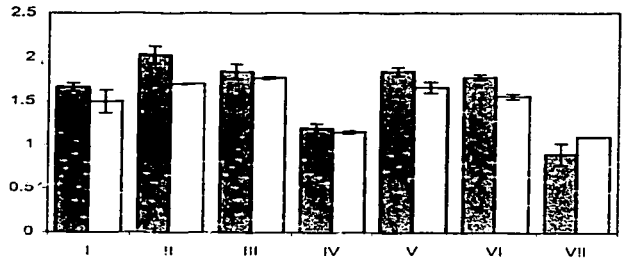

Figure 3. *Analysis of the mean hydrophobicity values of the seven transmembrane regions of COX III.* Hydrophobicity values for the seven transmembrane helices of COX III from *C. reinhardtii* and *Polytomella* sp. (white bars) were compared with the mean value of hydrophobicity of COX III from different organisms (grey bars). In Cr-COX III and Ps-COX III, mean hydrophobicity is reduced in transmembrane helix I (diminished by 10%, although not statistically significant), helix II (diminished by 16%), helix V (diminished by 10%) and helix VI (diminished by 12%) as compared to the mean values of <H> of plant and human transmembrane regions. Domains III and IV, although exhibiting diminished hydrophobicity by 4%, has no significant variation. The same is applicable to domain VII, whose hydrophobicity is increased by 22%, but the $\Delta G = 1.09$ kcal/mol for this transmembrane segment do not impede the import of the protein.

9. The allotopic expression of mitochondrial genes and the quest for human mitochondrial gene therapy

The first pathogenic mutations of the human mtDNA were described in the pioneering works of Holt *et al.*, [87] and Wallace *et al.*, [88]. Since then, more than 100 unique, pathogenic mutations of mtDNA have been reported [89].

Genetically, the mtDNA mutations fall into three categories 1) mutations in protein encoding genes; 2) mutations in structural RNA genes such as tRNAs and rRNAs; and 3) large-scale rearrangements (deletions or duplications) of the mtDNA. These alterations in human mtDNA result in a broad range of clinical outcomes.

Pathogenic mutations have been described for nearly all protein encoding mitochondrial genes [90]. Perhaps the most common are those associated with Leber's hereditary optic neuropathy (LHON). LHON presents as acute or subacute bilateral visual loss caused by severe bilateral optic nerve atrophy and is associated primarily with mutations in genes for subunits of NADH dehydrogenase [91]. Mutations in the ATP6 gene of the mtDNA are often associated with two syndromes: NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa) [92] and maternally-inherited Leigh's syndrome (MILS; subacute necrotizing encephalomyelopathy) [93,94]. Other protein coding mutations, in the mtDNA-encoded COX subunits, or cytochrome *b* of the bc_1 complex, present with a wide variety of clinical features, including progressive exercise intolerance, myopathy, encephalopathies, and multi-system disorders [95-102].

Several strategies to develop gene-based mitochondrial therapies for mitochondrial diseases have been reviewed [103-106]. Direct manipulation of the mtDNA is presently not possible. However DNA-protein conjugates can enter mitochondria via the protein import pathway, and chimeras of DNA attached to polypeptide presequences may be imported independently of its DNA length [107-109]. These techniques may eventually lead to the ability to correct mtDNA gene mutations. Another interesting approach for overcoming mutations in mtDNA-encoded proteins is to place a wild-type copy of the affected gene in the nucleus, and target the expressed protein to the mitochondrion to replace the defective mitochondrial protein. This approach requires the nuclear expression of genes that are normally localized in the mtDNA, their synthesis in the cytoplasm, and their successful import into the mitochondrial inner membrane.

Allotopic expression is defined as the functional activation of a gene in a cellular compartment different from its original location. Such "allotopic" expression of mitochondrial genes has been successfully performed in *S. cerevisiae* to overcome defects in mitochondrial bI4, the RNA maturase of the yeast mitochondrial matrix [110,111]; to study the functions of VAR1, a subunit of the mitochondrial ribosome [112]; and with ATP8 (subunit A6L), a small (48 aa) hydrophobic polypeptide of the F_0 component of the mitochondrial F_1F_0-ATP synthase [113]. The sequence of the *atp8* gene was genetically engineered for nuclear expression, and a sequence encoding a yeast MTS was artificially attached. This *atp8* gene was expressed, and its corresponding protein product was synthesized in the cytoplasm, imported

into the inner mitochondrial membrane, and assembled into a functional F_0 sector of F_1F_0-ATP synthase.

The fact that several typically mtDNA-encoded genes are nuclear-localized in some organisms, such as chlamydomonad algae, may facilitate the eventual allotopic expression of genes in human cells exhibiting mitochondrial diseases, especially those associated with defects in mitochondrial *atp6* genes [114], MILS and NARP [115], or those related with alterations in *cox* genes [95,99,100].

The allotopic expression of mitochondrial genes in the nucleus for gene therapy purposes, must be accompanied by the appropriate changes in codon usage of the transferred gene, and by the addition of an appropriate MTS. These two requirements may be necessary but not sufficient for successful allotopic expression. Human mitochondrial genes may also require additional alterations for allotopic expression. A decrease in the mean hydrophobicity of the protein product, particularly in those transmembrane stretches that are not highly conserved, and that are known to be non-critical for function may facilitate import of these highly hydrophobic proteins. Where appropriate the hydrophobicity of a protein could be diminished in the same regions where hydrophobicity has been reduced naturally in chlamydomonad algae. These approaches require a more detailed knowledge of the topology and function of the proteins of interest, more extensive site-directed mutagenesis of the allotopically expressed mitochondrial gene than previously considered, and a deep knowledge of the mechanisms of action of the coexpressed proteins.

For genes whose simple allotopic expression cannot produce an active mitochondria-localized protein, it may be plausible to co-express a gene that increases the import of such a protein. In yeast, some non-importable polypeptides can be expressed in the nucleus and imported efficiently into mitochondria when another gene is co-expressed [73]. The nature of these genes is being elucidated [116,117]. Overexpression of yeast karyopherin Pse1p/Kap121p stimulated the mitochondrial import of hydrophobic proteins [117]. In addition, hydrophobic mitochondrial proteins seem to be imported into mitochondria by means of ribosomes attached to the mitochondrial outer membrane. This scenario is analogous to the export of proteins to the endoplasmic reticulum [M. Corral-Debrinski, personal communication].

A number of genes encoding highly hydrophobic polypeptides have been retained with remarkable consistency in the mitochondrial genomes of a large number of eukaryotes. However, certain members of the chlamydomonad algae have relocated some of those genes to the nucleus. As we have described above, several structural transformations have accompanied chlamydomonad *cox2*, *cox3* and *atp6* genes on their way to the nucleus. Two important changes are the use of relatively long MTS and the reduction of hydrophobicty of their encoded protein products. This reduction of hydrophobicity is more notable in those

transmembrane regions that do not participate in proton translocation or in subunit-subunit interactions. Human therapies using allotopic expression of mtDNA-encoded genes, would typically require modifications to the genetic code, and the addition of the necessary transcriptional control signals and a region encoding an appropriate MTS. Further, one should consider reductions in the hydrophobicity of membrane-embedded helices that are not critical for function and assembly to facilitate the functional import of these proteins into mitochondria.

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Abbreviations

<H>	local hydrophobicity
*meso*H	mesohydrophobicity
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting sequence MTS
nt	nucleotides
TIM-TOM	translocases of the mitochondrial inner and outer
atp6	gene encoding subunit *a* (ATP6) of the F_1F_o-ATP synthase.
cox2	gene encoding subunit II (COXII) of cytochrome *c* oxidase.
cox3	gene encoding subunit III (COXIII) of cytochrome *c* oxidase.

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