Biochimica et Biophysica Acta 1817 (2012) 819–827

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

In *Polytomella* sp. mitochondria, biogenesis of the heterodimeric COX2 subunit of cytochrome *c* oxidase requires two different import pathways

Alejandra Jiménez-Suárez [1], Miriam Vázquez-Acevedo [1], Andrés Rojas-Hernández, Soledad Funes, Salvador Uribe-Carvajal, Diego González-Halphen *

Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, México 04510, D.F., Mexico

ARTICLE INFO

Article history:
Received 14 September 2011
Received in revised form 28 February 2012
Accepted 28 February 2012
Available online 7 March 2012

Keywords:
Polytomella sp.
Chlamydomonas reinhardtii
Mitochondrial targeting signal
COX2
Cytochrome *c* oxidase biogenesis

ABSTRACT

In the vast majority of eukaryotic organisms, the mitochondrial *cox2* gene encodes subunit II of cytochrome *c* oxidase (COX2). However, in some lineages including legumes and chlorophycean algae, the *cox2* gene migrated to the nucleus. Furthermore, in chlorophycean algae, this gene was split in two different units. Thereby the COX2 subunit is encoded by two independent nuclear genes, *cox2a* and *cox2b*, and mitochondria have to import the cytosol-synthesized COX2A and COX2B subunits and assemble them into the cytochrome *c* oxidase complex. In the chlorophycean algae *Chlamydomonas reinhardtii* and *Polytomella* sp., the COX2A precursor exhibits a long (130–140 residues), cleavable mitochondrial targeting sequence (MTS). In contrast, COX2B lacks an MTS, suggesting that mitochondria use different mechanisms to import each subunit. Here, we explored the *in vitro* import processes of both, the *Polytomella* sp. COX2A precursor and the COX2B protein. We used isolated, import-competent mitochondria from this colorless alga. Our results suggest that COX2B is imported directly into the intermembrane space, while COX2A seems to follow an energy-dependent import pathway, through which it finally integrates into the inner mitochondrial membrane. In addition, the MTS of the COX2A precursor is eliminated. This is the first time that the *in vitro* import of split COX2 subunits into mitochondria has been achieved.

1. Introduction

Cytochrome *c* oxidase or complex IV is the main final acceptor of the electron transfer chain in mitochondria, catalyzing oxygen consumption coupled to proton pumping. Cytochrome oxidase is composed of 10–13 subunits and its biogenesis involves both mitochondria- and nucleus-encoded polypeptides [1]. In the vast majority of eukaryotic organisms the three larger subunits of the complex, COX1 to COX3, are encoded in the mitochondrial genome. In contrast, in some chlorophycean alga including *Chlamydomonas reinhardtii* and several of its close relatives, the genes *cox2* and *cox3* are missing from the mitochondrial DNA [2-4]. These genes have migrated to the nucleus [5-8] and therefore, their corresponding proteins must be synthesized in the cytosol and imported by mitochondria in order to assemble complex IV. Furthermore, it was shown that in green algae the mitochondrial *cox2* gene was split into two genes, *cox2a* and *cox2b*, which migrated independently to the nucleus [7] and were inserted into different chromosomes [9,10]. The *cox2a* gene encodes COX2A, which is an equivalent to the N-terminal, membrane-bound half of an orthodox COX2 and contains two transmembrane segments (TMSs). COX2B is encoded in the nuclear *cox2b* gene and corresponds to the hydrophilic, C-terminal half of a conventional COX2 protein, *i.e.*, the region located in the intermembrane space (IMS). The chlorophycean COX2A and COX2B subunits contain unique C- and N-terminal extensions, respectively. These extensions, not present in orthodox COX2 subunits, are thought to stabilize the structure of the heterodimeric COX2 subunit of the algal cytochrome *c* oxidase complex [7] (Fig. 1A).

Given the structural differences between the COX2A and COX2B subunits of chlorophycean algae, we hypothesized that these proteins were imported into mitochondria through different pathways. Since COX2A exhibits a long, cleavable mitochondrial targeting sequence (MTS) plus two TMSs, it is probably imported through an energy-dependent pathway involving proteolytic processing of its MTS plus the insertion of the two TMSs into the inner mitochondrial membrane (IMM). By contrast, COX2B lacks an MTS and has no putative TMS; therefore it might be imported directly into the IMS. Here, we developed a protocol to isolate import-competent mitochondria from the colorless chlorophycean alga *Polytomella* sp. that allowed us to explore the *in vitro* mitochondrial import of the COX2A precursor and of the COX2B subunit and to suggest the possible routes that each of these proteins uses to reach its final location in the organelle.

* Corresponding author. Tel.: +52 55 5622 5620; fax: +52 55 5622 5611.
E-mail address: dhalphen@ifc.unam.mx (D. González-Halphen).
[1] Both authors contributed equally to this work.

0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2012.02.038

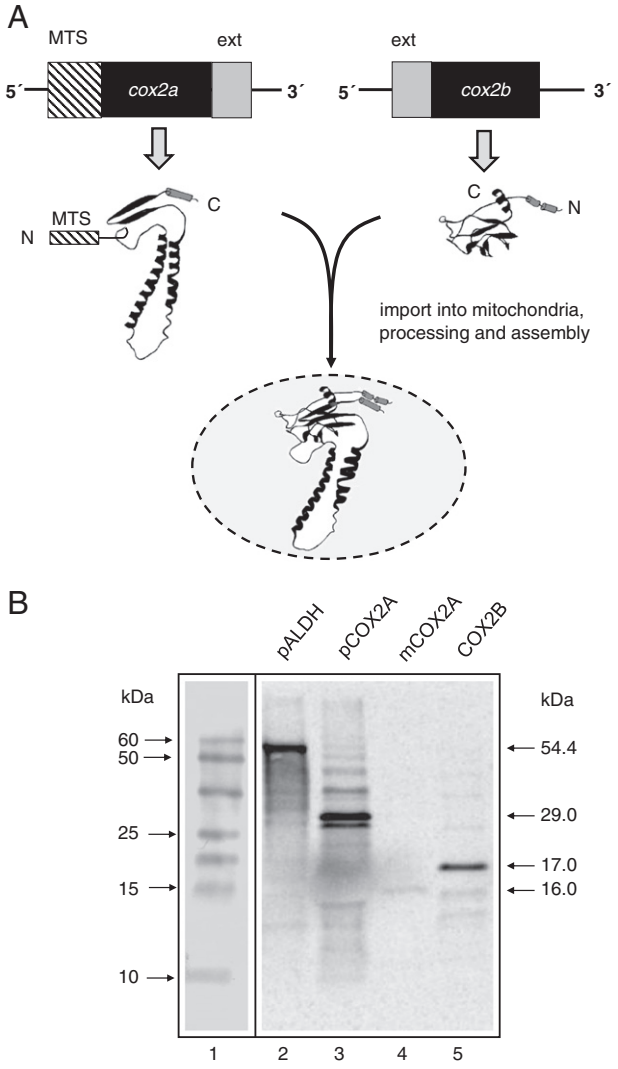


Fig. 1. In some chlorophycean algae, both nucleus-encoded COX2 subunits are synthesized in the cytosol and imported by mitochondria. Radiolabeled precursor proteins used in this study. A) In *Polytomella* sp. each COX2 subunit is encoded as an independent nuclear gene. When compared with canonical mitochondria-encoded COX2 subunits, COX2A presents a cleavable mitochondrial targeting signal (MTS) at the N-terminus and a C-terminal extension (shown in gray and marked as 'ext'). COX2B only exhibits a non-cleavable extension at the N-terminus. Both subunits assemble to form a mature COX2 within the cytochrome *c* oxidase. See text for details. B) Radiolabeled precursor proteins used in this study. Lane 1, molecular mass protein markers; lane 2, the rat ALDH precursor protein (pALDH of 54.4 kDa); lane 3, the algal COX2A precursor (pCOX2A of 29.0 kDa); lane 4, the mature COX2A protein (mCOX2A of 16.0 kDa); and lane 5, the COX2B protein (17.0 kDa).

2. Materials and methods

2.1. Strain and culture conditions

Polytomella sp. (198.80, E.G. Pringsheim) from the Sammlung von Algenkulturen (Göttingen, Germany) was grown as previously described [11]. Wistar rats and *Saccharomyces cerevisiae* strain W303 were used for isolation of mitochondria as described in Sections 2.3 and 2.4 respectively.

2.2. Cloning and sequencing of cDNAs encoding the precursor of COX2A, the mature form of COX2A and the COX2B subunit of Polytomella sp.

The *Polytomella* sp. cDNA sequences encoding the precursor of COX2A, the mature form of COX2A (GenBank accession number JN652595 that corrects the previous sequence AF305541), and COX2B (GenBank accession number AF305542) were amplified by PCR from a λ-ZAPII cDNA library [12]. The following primers were used: for the COX2A precursor, pcox2a_forward, 5′-TAT CTA GAA TGC TCG CCC AGC GTA TCT CT-3′ and pcox2a_reverse, 5′-CGC GAG CTC TTA CTT GGT GAT AGT CTT CTC-3′; for the mature COX2A, mcox2a_forward, 5′-GCC TGC AGA TGG AGG CCC CTG TCG CTT GGC AG-3′ and pcox2a_reverse; for COX2B, cox2b_ forward, 5′-TAC TGC AGA TGT CGG ATG CTA AGG ACC AG-3′ and cox2b_ reverse, 5′-GCG GAG CTC TTA CTG AAT CCA CTT CTT GAC-3′. The amplified PCR-products were fractionated on 1% agarose gels, purified with the QIAquick gel extraction kit (Qiagen), and cloned into pGEM®-T (Promega). The precursor *cox2a* insert was released with the restriction enzymes *Xba*I and *Sac*I, the mature *cox2a* and the *cox2b* inserts were released with *Pst*I and *Sac*I, and all of them were transferred to the pSP64polyA vector in order to perform their synthesis *in vitro* for import experiments.

2.3. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated as described by Hogeboom et al. [13] with some modifications [14]. Briefly, male Wistar rats weighing 120 to 160 g were fasted overnight and killed by decapitation; the liver was rapidly extracted and immersed in 40 ml of cold 70 mM sucrose, 10 mM HEPES, pH 7.4 buffer. Livers were minced and homogenized in a Potter-Evelheim homogenizer with a Teflon pestle. Mitochondria were isolated from the homogenate by differential centrifugation [13]. Protein concentration was determined by biuret [15]. Mitochondria were used within 3 h of preparation.

2.4. Isolation of yeast mitochondria

Yeast were grown in YPLac media (1% yeast extract, 2% peptone, 2% lactate, 0.1% glucose) under agitation at 200 rpm in a New Brunswick shaker in a constant temperature room at 30 °C, about 16 h to the late logarithmic growth phase. Mitochondria were isolated by differential centrifugation as in Herrmann et al. [16]. Protein was determined by biuret [15]. Mitochondria were always used within 3 h of preparation or frozen in liquid nitrogen and stored at −70 °C for further use.

2.5. Isolation of import-competent mitochondria from Polytomella sp.

The technique to isolate mitochondria from *Polytomella* sp. was based on a protocol designed for *S. cerevisiae* [17]. All steps were carried out at 4 °C. Cells were collected at 1600×*g* for 5 min at 4 °C. The pellet was resuspended in buffer A (0.375 M sorbitol, 0.2 M mannitol, 10 mM Tris-maleic, pH 6.8, 0.1% fatty acid-free bovine serum albumin) and centrifuged again under the same conditions. The cells were resuspended in buffer A containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0 mM EDTA pH 8.1 in order to reach 0.5 g WW/mL. Cells were broken mechanically with three strokes in a glass–glass Dounce tissue homogenizer, and centrifuged at 300×*g* for 10 min. The supernatant was collected and centrifuged at 15,000×*g* for 10 min. The pellet was washed with buffer A and both centrifugation steps were repeated. The final pellet, containing mitochondria was resuspended carefully with a fine-tip painting brush in 200 μL of buffer A. Protein was quantified using the biuret method [15]. Oxygen uptake was measured in an oxymeter (Strathkelvin/Warner Instruments) to verify that mitochondria exhibited respiratory control (with typical values varying between 1.5 and 3.0). Oxygen consumption by mitochondria (0.2 mg of protein) was measured at 30 °C in a final volume of 100 μL in a buffer containing 0.6 M mannitol, 5 mM Mes, pH 6.8, 30 mM H_3PO_4, 200 μM $MgCl_2$ and 2 mM NAD^+, in the presence of either 128 mM ethanol or 10 mM malate as substrates and 200 μM ADP.

2.6. In vitro protein synthesis

The cDNAs encoding the COX2A precursor and COX2B described in Section 2.2 and rat aldehyde dehydrogenase (ALDH, [18]) were used as templates to synthesize the corresponding radiolabeled proteins using the reticulocyte lysate TNT® (T7 or SP6) quick-coupled transcription/translation system (Promega) in the presence of 0.38–0.75 MBq of L-[^{35}S]-methionine (Perkin Elmer) in the conditions suggested by the manufacturer. In the case of the mature COX2A, the TNT® coupled wheat germ extract system (Promega) was used. The mixtures were incubated for 90 min at 30 °C before an excess of non-radioactive methionine was added and further incubated for 10 min on ice. When the COX2A precursor internalization was followed, the *in vitro* synthesis reaction mixture was added directly to isolated mitochondria. In contrast, in order to track the mitochondrial import of COX2B, the *in vitro*-synthesized protein was precipitated with saturated ammonium sulfate on ice, centrifuged at 12,000×*g* for 10 min at 4 °C, and the pellet was resuspended in 10 mM Tris-maleic buffer pH 6.8 containing 8 M urea [19].

2.7. In vitro protein import into rat mitochondria

Isolated rat mitochondria (62 μg of mitochondrial protein) were incubated with [^{35}S]-methionine-labeled proteins in a total volume of 125 μL in 3% fatty acid-free bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH pH 7.2, 2 mM ATP, 10 mM malate–glutamate, 12.5 U creatine phosphokinase, and 10 mM phosphocreatine, for 30 min at 25 °C. An aliquot of 60 μL was removed and treated with proteinase K (300 μg/mL) 30 min on ice, to digest the proteins that were not internalized by mitochondria. PMSF was added (5 mM final concentration) to stop the proteinase K digestion. A second 125 μL reaction was set as before but with the addition of 1 μL AVO stock solution (8 mM antimycin A, 0.1 mM valinomycin and 2 mM oligomycin), to prevent ΔΨ-dependent protein import. Mitochondria were re-isolated by centrifugation, washed, solubilized in sample buffer and resolved by SDS-PAGE [20]. Proteins were transferred to a PVDF membrane and the radioactive label was analyzed using either a Storm or a Typhoon 9400 phosphorimager system (GE Healthcare) and the corresponding images were digitalized.

2.8. In vitro protein import by yeast mitochondria

Import reactions were performed as described by Herrmann et al. [21]. Briefly, isolated yeast mitochondria (300 μg of protein) were incubated 5 min at 25 °C at a concentration of 1 mg prot/mL in 0.6 M sorbitol, 80 mM KCl, 10 mM magnesium acetate, 2 mM KH_2PO_4, 2.5 mM EDTA, 2.5 mM $MnCl_2$, 50 mM Hepes pH 7.2, 2 mM ATP, 4 mM NADH and fatty acid-free bovine serum albumin (1 mg/mL). Then 10 μL of [^{35}S]-methionine-labeled proteins were added and incubated 25 min at 25 °C. Import reactions were stopped immersing the sample test tubes in ice.

After 10 min in ice, a 100 μL aliquot was removed and treated with proteinase K (100 μg/mL) and left for 30 min on ice, to digest the proteins that were not internalized by mitochondria. PMSF was added (2 mM final concentration) to stop the proteinase K digestion. Samples were centrifuged at 16,000×*g* for 15 min and the pellet washed with 0.6 M sorbitol, 20 mM Hepes pH 7.4. A second 100 μL aliquot was subjected to proteinase K as described above, except for the addition of 1 μL AVO which depletes the ΔΨ. Mitochondria were re-isolated by centrifugation, washed and solubilized in sample buffer, resolved by SDS-PAGE and transferred to a PVDF membrane and analyzed as described in Section 2.7.

2.9. In vitro protein import into Polytomella sp. mitochondria

Polytomella mitochondria (1 mg of prot/mL) were incubated in the presence of the *in vitro*-synthesized labeled protein of interest. Import reactions were carried out with 10 μL of [^{35}S]-methionine-labeled proteins at 25 °C for 30 min in import buffer (375 mM sorbitol, 200 mM manitol, 10 mM Tris-maleic pH 6.8, 1 mg/mL fatty acid-free bovine serum albumin, 10 mM H_3PO_4, 200 μM $MgCl_2$, 0.75 mg/mL methionine, 2 mM ATP, 2 mM NAD^+, 10 mM ethanol or 10 mM malate, 10 mM phosphocreatine, and 25 units of creatine kinase. When indicated, AVO was added or the reaction was carried out in the presence of 2 mM 1,10-ortophenantroline. To disrupt the outer mitochondrial membrane by osmotic shock, 20 mM Hepes or 20 mM Tris–HCl pH 7.2 was used. The internalization of polypeptides was estimated as resistance to degradation by proteinase K (50 μg/mL, final concentration). To stop the action of the protease, 2 mM PMSF was added to the reaction mixture, which was then incubated on ice for 10 min. Reactions were centrifuged at 12,000×*g* for 5 min at 4 °C, the pellet was washed with 900 μL of 0.6 M sorbitol, 10 mM Hepes pH 7.2 and centrifuged again under the same conditions. Where indicated, carbonate extraction of peripherally associated membrane proteins was carried out after the import reaction was completed [22]. In all cases, the final pellet was resuspended in sample buffer and samples were analyzed as described in Section 2.7.

2.10. Sequence analysis in silico

Protein sequences were obtained from ENTREZ at the NCBI server (www.ncbi.nlm.nih.gov), and multiple alignments carried out with ClustalW [23]. TMSs were established with the transmembrane topology and signal peptide predictor Phobius (http://phobius.sbc.su.se/) and all were adjusted to 25 residues. Mean hydrophobicity for each TMS was calculated using the Kyte and Doolittle hydrophobicity scale [24].

3. Results

3.1. Isolation and characterization of import-competent mitochondria

In order to carry out experiments aimed at understanding the mechanism of mitochondrial import used by COX2A and COX2B respectively, we synthesized four radiolabeled proteins, the rat ALDH precursor protein, the algal COX2A precursor, the mature COX2A protein and the COX2B protein in the conditions described in Section 2.6. The obtained products and their molecular masses are shown in Fig. 1B.

We then developed a protocol (detailed in Section 2.5.) to isolate intact mitochondria from *Polytomella* sp. with a measurable respiratory control. The algal mitochondria consumed oxygen in the presence of malate, succinate or ethanol (data not shown) and exhibited variable respiratory controls ranging from 1.6 to 3.0, depending on the preparation. In order to ascertain if the mitochondrial fraction was import-competent, we used the rat ALDH precursor (54 kDa), a well-characterized protein that is targeted to and processed in the mitochondrial matrix of rat liver mitochondria [18]. In the absence of mitochondria, the *in vitro* synthesized [^{35}S]-labeled precursor was readily digested by proteinase K (Fig. 2A, lane 2, upper panel). Import was assayed for 30 min in mitochondria prepared from three different sources: rat liver, yeast, and *Polytomella* sp. The ALDH precursor was internalized into the mitochondria from all three sources, as evidenced by the protection of the precursor from externally added proteinase K (Fig. 2A, lane 4). In rat liver and yeast mitochondria the internalized precursor (labeled pALDH) was processed to a smaller, mature form (labeled mALDH) (Fig. 2A, compare lanes 3 and 4, upper and middle panels). The mitochondria from *Polytomella* sp. were also capable of protecting the precursor against the activity of

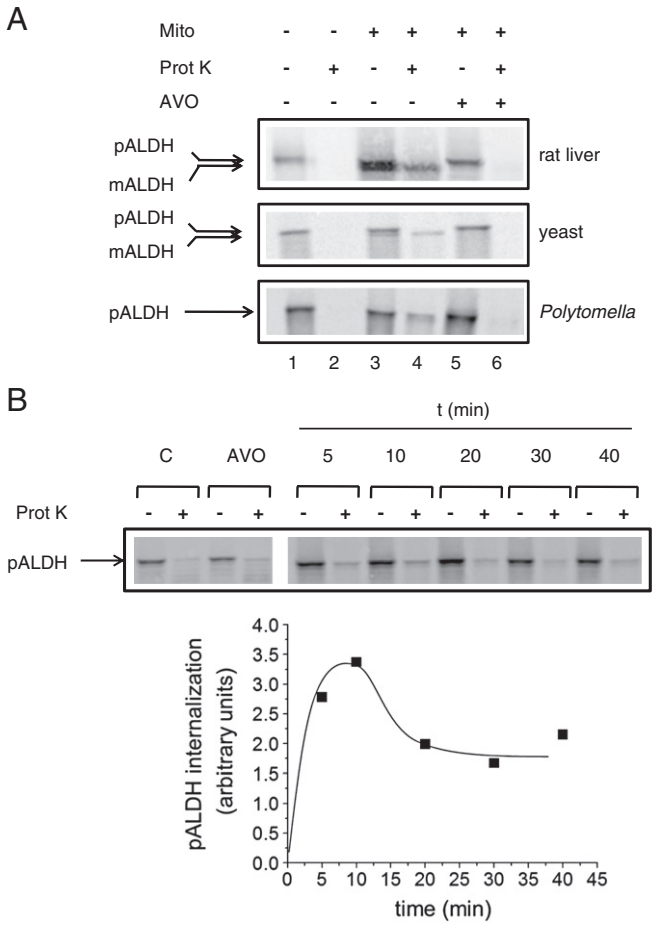


Fig. 2. The rat ALDH precursor can be imported but not processed by *Polytomella* mitochondria. A) The radiolabeled rat ALDH precursor protein was imported into isolated mitochondria from rat, yeast and *Polytomella* sp. as indicated. The *in vitro* [^{35}S]-labeled precursor (lane 1) was incubated with proteinase K (lane 2) to verify that it could be completely digested when not imported. ALDH precursor (pALDH) was processed to a mature form (mALDH) only when the import reaction was performed in mitochondria from rat liver (lanes 3 and 4, upper panel) and yeast (lanes 3 and 4, middle panel). However when mitochondria from *Polytomella* sp. were used, the processing to the mature form was not observed (lanes 3 and 4, lower panel). In all cases, the import reaction was dependent on the mitochondrial membrane potential as it was inhibited by the addition of AVO (lanes 5 and 6). B) ALDH was imported into *Polytomella* sp. mitochondria incubated during the indicated times before the reaction was treated with or without proteinase K. The precursor alone (C) or in a reaction where the mitochondrial membrane potential was depleted (AVO) was completely digested by proteinase K. Below, quantification by densitometry of the imported ALDH.

externally-added protease, indicating that these mitochondria internalized the protein (Fig. 2A, lane 4), however, in *Polytomella* sp. mitochondria the precursor form was not processed, as evidenced by the lack of a mature form (Fig. 2A lanes 3 and 4, lower panel), *i.e.* the algal mitochondrial processing protease seems not to recognize the cleavage site that removes the MTS of the mammalian ALDH. In mitochondria from all three species tested, the import of ALDH was ΔΨ-dependent, since addition of AVO abolished protection from proteinase K (Fig. 2A lanes 5 and 6). In order to evaluate the efficiency of the *Polytomella* sp. organelle to import ALDH, a time course of mitochondrial uptake of ALDH was performed (Fig. 2B). It was observed that import was maximal in the first 5 to 10 min, and decreased at longer incubation times (20 to 40 min), indicating that isolated algal mitochondria are unable to sustain the ΔΨ-dependent internalization of this polypeptide for long periods. The decrease in intensity of the protease-protected ALDH bands observed at longer times (20 min and later) suggests that in *Polytomella* sp. ALDH may be subjected to high turnover once it has reached the mitochondrial matrix.

3.2. In vitro import of the COX2A precursor

In order to explore the COX2A import mechanism in *Polytomella* sp. mitochondria, we measured the uptake of the [^{35}S]-labeled COX2A precursor in isolated mitochondria (Fig. 3A). The COX2A precursor was captured and it was proteolytically processed to its mature form (Fig. 3A, lanes 3 and 4) in a ΔΨ-dependent process that was abolished by the addition of AVO (Fig. 3A lanes 5 and 6). Only a fraction of the mature form of COX2A became resistant to protease digestion (6% of the precursor that was added to the assay, as judged by densitometric analysis), whereas import reactions conducted in the presence of AVO fully sensitized COX2A to protease digestion. The mature COX2A form was not observed when the reaction was performed in mitochondria incubated in Tris buffer, where they underwent a hypoosmotic shock-mediated disruption of the outer membrane (Fig. 3A, lanes 7 and 8). The use of GTP (2 mM) or a mixture of ATP and GTP (2 mM each) did not improve the import of COX2A observed in the presence of 2 mM ATP (data not shown). The precursor and mature forms of the algal COX2A subunit were identified by their molecular masses using the corresponding *in vitro* synthesized [^{35}S]-labeled polypeptides as molecular weight standards (Fig. 3B).

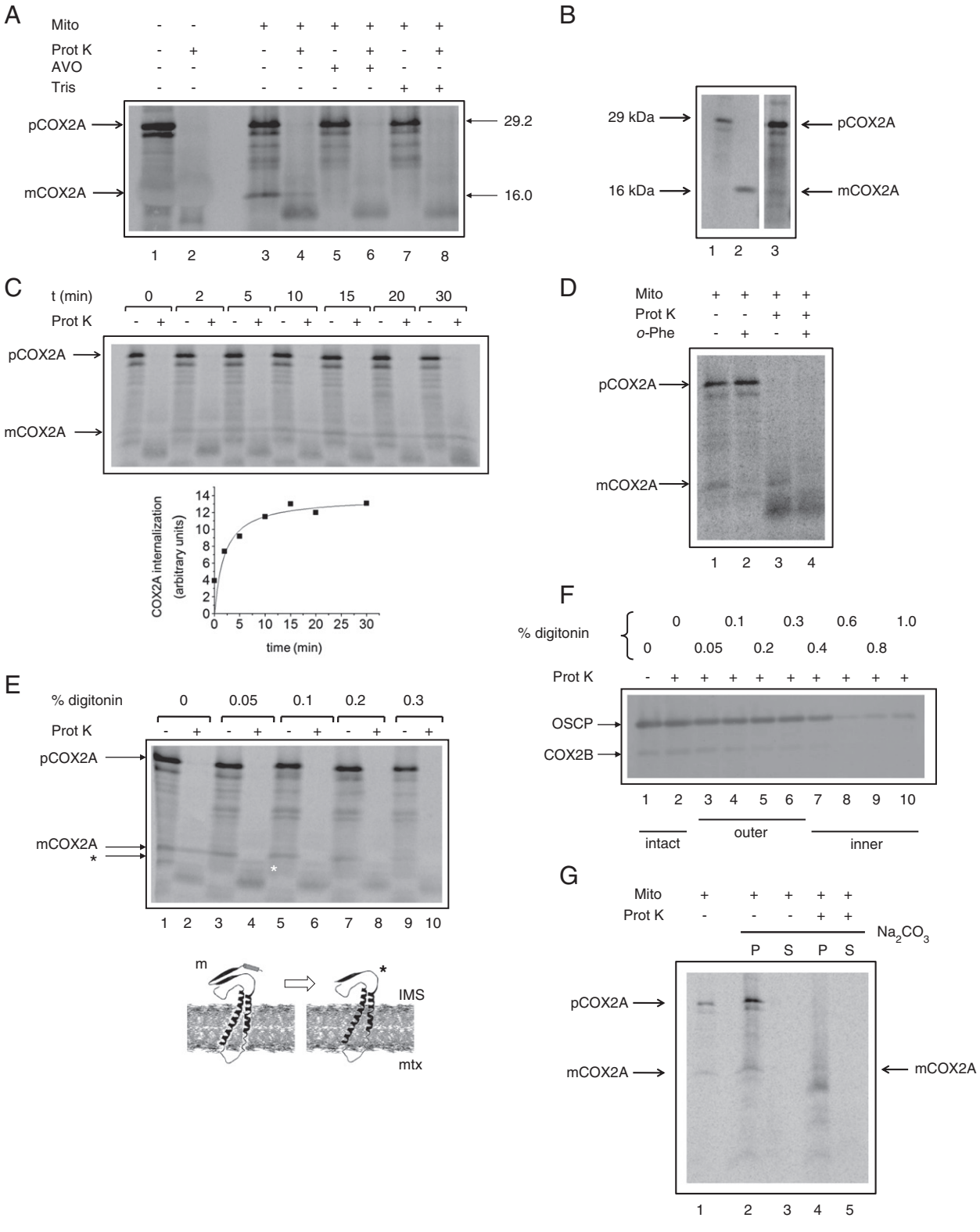
A time course of COX2A uptake by algal mitochondria was also performed. The band corresponding to mature COX2A was maximal in the first 5 to 15 min, and then it remained constant (Fig. 3C). A parallel oxymetry experiment was performed using the same mitochondrial preparation incubated in import buffer and the changes in respiratory control were measured. The original respiratory control of 2.2 dropped down to 1.6 after 15 min and to 1.3 after 30 min. As suggested by these data and the ALDH and COX2A uptake kinetics, the isolated algal mitochondria do not seem to remain coupled for long.

The mitochondrial matrix protease (MPP) has been previously reported to be inhibited by 1,10-ortophenantroline [25]. When the COX2A import reaction was carried out in the presence of this iron-chelating agent (Fig. 3D) the mature form of COX2A became

Fig. 3. Import and processing of COX2A precursor requires energized mitochondria from *Polytomella* sp. A) COX2A precursor (pCOX2A, 29.2 kDa) was synthesized *in vitro* and treated with proteinase K (Prot K) to verify its sensibility to the protease (lanes 1 and 2). The band observed just below the precursor might be an incomplete COX2A precursor polypeptide resulting from the initiation of a downstream AUG codon. When isolated mitochondria were incubated with the COX2A precursor the appearance of the mature form (mCOX2A, 16.0 kDa) was observed (lanes 3 and 4). Approximately 6% of the mature product was also resistant to the action of proteinase K (lane 4). This mature form was not present when the samples were treated with AVO (lanes 5 and 6) or in mitochondria with a disrupted outer membrane (lanes 7 and 8). Even lanes show reactions treated with proteinase K (Prot K). B) Estimation of the molecular masses of the precursor (pCOX2A) and of the mature (mCOX2A) COX2A subunit. Lane 1, the 29 kDa COX2A precursor synthesized *in vitro*; lane 2, the 16 kDa mature form of COX2A synthesized *in vitro*. Lane 3, import reaction of COX2A into *Polytomella* sp. mitochondria showing the precursor (pCOX2A) and the mature form (mCOX2A). C) Time course of COX2A internalization into *Polytomella* sp. mitochondria and its quantification by densitometry (below). D) The presence of 1,10-orthophenantroline during the import of the COX2A precursor inhibits the formation of the mature form of COX2A. Lanes 1 and 3 show control import reactions, lanes 2 and 4 show reactions in the presence of 1,10-orthophenantroline (*o*-Phe); proteinase K was added to samples loaded in lanes 3 and 4 to digest unprotected material. E) COX2A is inserted into the mitochondrial inner membrane after the import reaction. A COX2A import reaction was distributed in different aliquots that were incubated with increasing amounts of digitonin. The asterisk (*) indicates the putative partial degradation product of COX2A after limited proteolysis (see scheme below). Even lanes are samples treated with proteinase K. F) Western blot analysis of *Polytomella* sp. mitochondria treated with proteinase (Prot K) at increasing concentrations of digitonin, decorated with anti-OSCP (matrix) and anti-COX2B (IMS) antibodies. Lanes 1–2, intact outer and inner membranes; lanes 3–6, permeabilized outer membrane and intact inner membrane; lanes 7–10, the inner membrane was also permeabilized. G) The mature form of COX2A is recovered in the membrane fractions after carbonate extraction. Lane 1 shows a control import reaction, lanes 2 to 5, fractions recovered after Na_2CO_3 treatment (P, pellet; S, supernatant).

undetectable, regardless of whether proteinase K was added (Fig. 3D, lanes 2 and 4). This strongly suggests that the MTS of the COX2A precursor is partially or totally processed in the mitochondrial matrix.

In order to identify the mitochondrial sub-compartment where the imported COX2A resides, the samples were treated with increasing amounts of digitonin before the addition of proteinase K (Fig. 3E). After the import reaction, a fraction of the newly imported COX2A was protected from the protease (Fig. 3E, lane 2: mCOX2A). This mature form disappeared as soon as 0.5% digitonin was added leading to the appearance of a slightly smaller band (Fig. 3E lane 4 indicated with an asterisk). Probably this band is an intermediate form where the second transmembrane domain of COX2A has been inserted into the inner membrane, leaving the C-terminus of COX2A exposed to the IMS. This C-terminus would be then digested by proteinase K after dissolving the outer membrane (Fig. 3E, bottom scheme). In the presence of increasing amounts of digitonin, all forms disappear



gradually, including those presumably found within the matrix (Fig. 3E lanes 6, 8 and 10). We explored the integrity of the outer and inner mitochondrial membranes in *Polytomella* sp. mitochondria using antibodies against the oligomycin sensitivity conferring protein (OSCP), a subunit of the ATP synthase that is exposed to the matrix space, and against the native, mature COX2B subunit bound to cytochrome *c* oxidase, a protein that is exposed to the IMS and interacts with soluble cytochrome *c* [7]. Concentrations of 0.05% to 0.3% digitonin allowed the digestion of COX2B. At higher digitonin concentrations the inner membrane was also disrupted, as evidenced by the protease K-mediated digestion of OSCP (Fig. 3F, lanes 7–10).

Carbonate extraction has been widely used as a criterion to identify integral membrane proteins, which are usually not extracted by this alkaline treatment [26]. After internalizing COX2A for 30 min (Fig. 3G, lane 1) and treatment with externally-added proteinase K where indicated, mitochondria were subjected to Na_2CO_3 extraction, and the resulting supernatants and pellets analyzed. Bands corresponding to the mature form of COX2A were found only in the membrane fraction pellets, both in intact mitochondria and in mitochondria exposed to proteinase K (Fig. 3G, lanes 2 and 4). In contrast, no traces of COX2A were present in the corresponding supernatants (Fig. 3G, lanes 3 and 5). These results reinforce the idea that the mature form of COX2A is embedded in the IMM.

3.3. In vitro import of the COX2B subunit

The presence of a heterodimeric COX2 subunit composed of COX2A and COX2B in the isolated cytochrome c oxidase from *Polytomella* sp. has been previously demonstrated [7]. In order to assemble this heterodimeric COX2, *Polytomella* mitochondria need to import both the COX2B and the COX2A subunits. Thus, we decided to quantitate the import of [^{35}S]-labeled COX2B into isolated *Polytomella* sp. mitochondria. In contrast with COX2A, the COX2B precursor was internalized into mitochondria without being proteolytically processed (Fig. 4A, lanes 3–6). Furthermore, COX2B readily became resistant to the action of externally added proteinase K and it was imported even in the presence of AVO (Fig. 4A lane 6). These results suggest that COX2B is not translocated through the IMM, but instead is directly sent to the IMS from the mitochondrial outer membrane. In contrast to COX2A, a time course of the COX2B import reaction suggests that the fraction of protein internalized increased for at least 30 min (Fig. 4B). Also, in contrast to COX2A internalization, COX2B was only imported in an unfolded state after re-suspension in 8 M urea.

In order to confirm that COX2B remained in the IMS and it was not translocated further to the matrix, we tested in digitonin-treated mitochondria the sensitivity of the protein to proteinase K (Fig. 4C). The detergent was added before the import reaction. At low concentrations (0.05%), the detergent disrupts selectively the outer mitochondrial membrane without damaging the IMM (Fig. 3F). As soon as this occurs, the COX2B subunit is no longer internalized and becomes susceptible to proteinase K degradation. This is in accordance with the idea that the *in vitro* imported COX2B protein resides in the IMS, *i.e.* the compartment where COX2B is expected to function *in vivo*, receiving electrons from soluble cytochrome *c*. To gain insights on whether this protein is imported in an oxidation-driven mechanism by the mitochondrial disulfide relay in the IMS [27], we also explored the effect of reducing agents on the import process of COX2B. We followed the kinetics of COX2B internalization in the presence and absence of dithiothreitol (DTT). The presence of DTT increased slightly (10%) but consistently the import of COX2B (Fig. 4D).

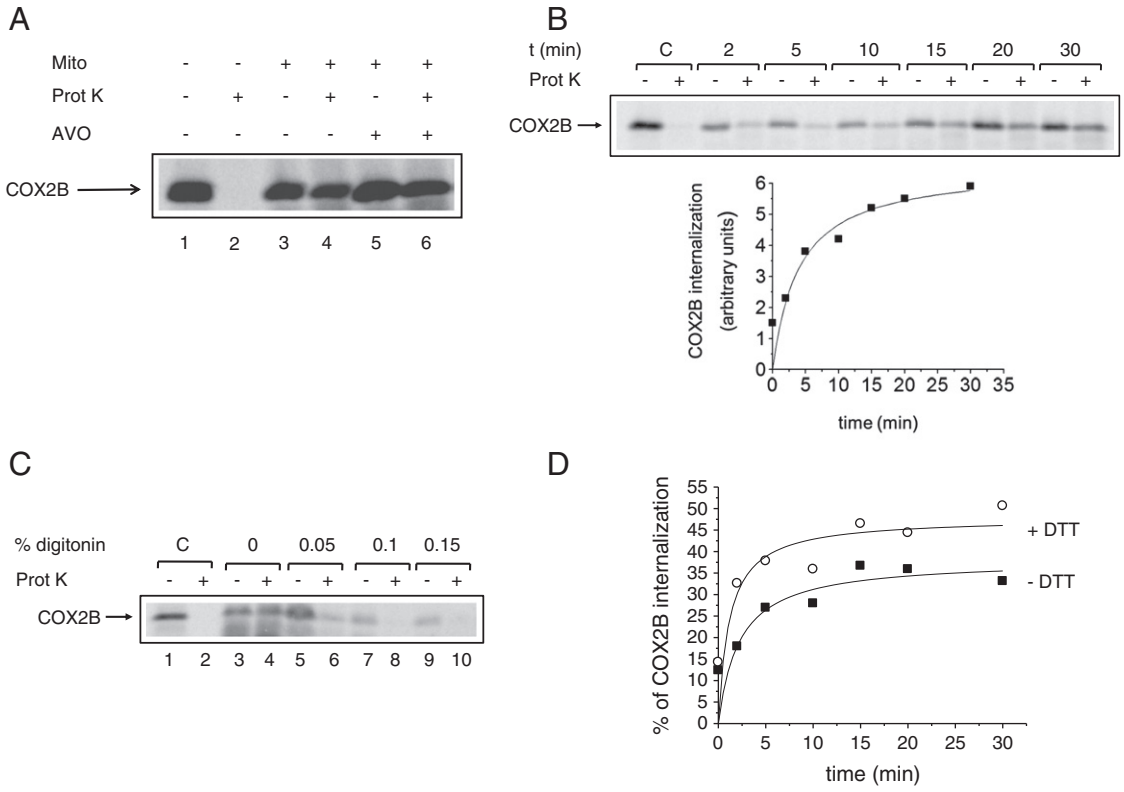


Fig. 4. The COX2B polypeptide is inserted directly into the IMS of *Polytomella* sp. mitochondria. A) Import of COX2B into isolated mitochondria. Lanes 1 and 2 show the radioactively labeled protein and its complete degradation upon incubation with proteinase K (Prot K). Lanes 3 and 4 show import reactions in the presence of a membrane potential (−AVO) while lanes 5 and 6 are in the absence of $\Delta\psi$ (+AVO). Proteinase K (Prot K) was added to samples in even lanes to digest non translocated material. B) Time course of COX2B internalization into *Polytomella* sp. mitochondria and below, the quantification by densitometry of the imported COX2B. C) Dependence of COX2B internalization on the integrity of the outer mitochondrial membrane. D) Import of COX2B is influenced by the presence of reductive agents. Quantification by densitometry of the import kinetics of COX2B in the presence (white circles) or absence (black boxes) of DTT.

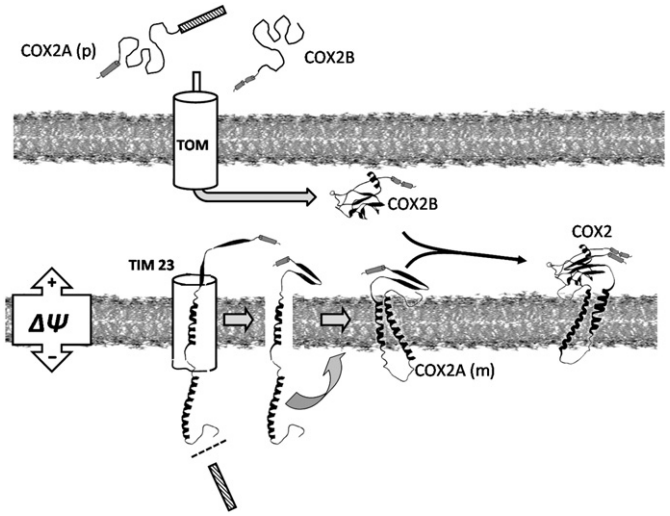


Fig. 5. Model depicting the two different import pathways involved in the biogenesis of the heterodimeric COX2 subunit of cytochrome *c* oxidase in *Polytomella* sp. mitochondria. COX2A and COX2B follow specific and independent import pathways before their assembly into the functional cytochrome *c* oxidase complex. COX2A contains an MTS, which directs it to the matrix and probably involves more than one translocase in order to achieve its final membrane topology. In contrast, COX2B is directed into the inter-membrane space. See text for details.

4. Discussion

In most aerobic eukaryotes, the *cox2* gene is located in the mitochondrial DNA and the COX2 subunit is synthesized by mitochondrial ribosomes and co-translationally inserted in the IMM in a $\Delta\Psi$-dependent process [28]. This insertion is mediated by the Oxa1 translocase [29,30]. Mitochondrion-encoded COX2 usually lacks a signal sequence, but in *S. cerevisiae* it is synthesized as a precursor that is processed during biogenesis by removal of a 15 amino acid segment [31-33].

In some species, the gene *cox2* gene has migrated to the nucleus. Many legumes contain orthodox, mitochondrial *cox2* genes, while others show nucleus-localized *cox2* genes and yet others exhibit both the mitochondrial and the nuclear versions of *cox2* [34]. The different localizations of *cox2* genes in legumes, illustrates the fact that mitochondrial gene migration to the nucleus is an ongoing evolutionary process. It has been shown that mitochondrial gene relocation is usually accompanied by changes in codon usage patterns and in the actual nucleotide sequences of the genes involved [35]. Also, in many cases, the corresponding protein sequences exhibit lower hydrophobicity than their mitochondrial DNA-encoded counterparts [5,7,36,37]. Specifically, Daley et al. [36] demonstrated that in soybean COX2, two amino acid changes in the first TMS, engineered to decrease the overall hydrophobicity of the protein, in addition to the presence of a MTS, are sufficient to allow *in vitro* import of the protein by isolated mitochondria. Experiments carried out with an allotopically expressed COX2 subunit in yeast, in which the mitochondrial *cox2* gene was engineered to be expressed from a cytosolic vector, also show that replacing a hydrophobic residue to increase polarity in the first transmembrane stretch of COX2 results in functional mitochondrial import *in vivo* [38].

In the case of legumes where the mitochondrial *cox2* gene has migrated to the nucleus, the gene has been relocated as a single unit. In contrast, in chlorophycean algae the original gene was split into two *cox2* genes, *cox2a* and *cox2b*, which then migrated independently to the nucleus [39]. Each of these two genes codes for a subunit that complements the other to form a functional COX2 heterodimeric protein. A series of evolutionary events may have taken place before the appearance of split, nucleus-encoded *cox2* genes: *i)* an insertion in an ancestral mitochondrial, orthodox, *cox2* gene which did not disrupt COX2 function, *ii)* splitting of the mitochondrial *cox2* gene into *cox2a* and *cox2b*, *iii)* independent and sequential migration of the fragmented genes to different nuclear chromosomes, *iv)* insertion and functional activation of the re-localized genes, and *v)* progressive elimination of the remaining *cox2a* and *cox2b* mitochondrial copies. The remnants of this process are the unique C-terminal and N-terminal extensions located in each of the COX2A and COX2B subunits that are not present in any orthodox mitochondrial COX2 subunit.

This work provides the first experimental data on the import of each of the two algal split COX2 subunits into isolated mitochondria. A prerequisite for these experiments was to have a preparation of isolated organelles capable of building an electrochemical gradient across the IMM when in the presence of a respiratory substrate. Few *in vitro* protein import experiments have been carried out with isolated mitochondria from chlorophycean algae. Nurani et al. [40] developed an import system using isolated mitochondria from *C. reinhardtii*, and studied the import of several precursor proteins of the green alga: the alpha and beta subunits of ATP synthase and the Rieske FeS protein of the bc_1 complex. The conditions needed to obtain import-competent mitochondria from *C. reinhardtii* have been detailed [41]. *Polytomella* sp. is closely related to *C. reinhardtii*, but lacks chloroplasts and a cell wall, and thus it presents many technical advantages for the isolation of coupled mitochondria. Conditions to isolate *Polytomella* sp. mitochondria capable of building a $\Delta\psi$ included absence of potassium, low concentrations of magnesium, relatively high concentrations of phosphate and addition of NAD^+. NAD^+ addition was attempted after observing that several NAD^+-dehydrogenases in the mitochondrial matrix were working at low rates, which led us to hypothesize that the matrix NAD^+ pool might have been lost during mitochondrial isolation. Indeed, a NAD^+ specific transporter has been described in yeast, mammalian and plant mitochondria [42-44]. NAD^+ addition did result in recovery of dehydrogenase activities.

Mining the *C. reinhardtii* genome [9], several of the putative participants of the algal mitochondrial import machinery were identified [45,46]. The vast majority of the main translocation components characterized in yeast [47,48] seem to be present in *C. reinhardtii*, including the TOM and SAM complexes of the outer mitochondrial membrane; chaperones of the IMS (small Tims) and components of the disulfide relay pathway (Mia40 and Erv1); and in the IMM Tim22, Tim23 and Oxa1. Given the close phylogenetic relationship between *Polytomella* sp. and *C. reinhardtii*, we suggest that these algae contain similar import machineries.

Most nucleus-encoded mitochondrial proteins contain an MTS. In general 30–70 residues are enough to direct a mitochondrial protein to the organelle, although a long MTS of 136 residues is present in the soybean nucleus-encoded COX2 precursor [49]. Long MTS are also present in several *C. reinhardtii* nucleus-encoded mitochondrial proteins, such as ATP6 (107 amino acids), COX3 (110 residues), COX2A (130 amino acids), NAD3 (160 residues) and NAD4L (133 amino acids) [45]. These long MTS form amphiphilic α-helices that could be potentially processed more than once, and its first 30 to 50 residues could function as matrix-targeting signals [50]. Although several bands that could correspond to intermediates were consistently observed between the precursor and mature forms of COX2A, these bands were not eliminated by the presence of AVO or by preincubation with 1,10-ortophenantroline.

Our data do not allow us to distinguish between various possible routes of COX2A insertion into the IMM. Nevertheless, we favor a mechanism in which Tim23, after fully translocating the MTS and the first TMS of COX2A to the matrix, recognizes a stop transfer signal within the second TMS and thereby inserts it laterally into the inner membrane. Afterwards, the N-terminal part of the mature COX2A would be translocated back in an export-like reaction (probably through the Oxa1 machinery) in order to achieve the functional N-

out C-out topology of COX2A (Fig. 5). This import mechanism is similar to the import route proposed for the nucleus-encoded soybean COX2 precursor [49]. A second, different route could involve a direct, lateral incorporation of both TMSs of COX2A to the IMM also mediated by Tim23. An alternative third route would imply the complete translocation of COX2A by Tim23 to the mitochondrial matrix and the subsequent insertion of both TMSs into the inner membrane. The delivery of the full COX2A to the matrix would ensure that the protein will then be able to use the same translocases it used when it was encoded in the mitochondrial genome. A fourth route mediated by different IMM protein translocation components cannot be discarded: the insertion of COX2A through the carrier translocase of the inner membrane involving the small Tims and Tim22, following the route of biogenesis characteristic of the membrane-bound transport carriers [51,52]. This would imply the insertion of a loop containing the two TMSs of COX2A into the IMM. Alternatively, a portion of the MTS of COX2A is recognized by TIM23 as a TMS and is laterally inserted into the IMM, the two TMSs of the mature form of COX2A are then inserted through the carrier translocase of the inner membrane involving TIM22. This would imply the insertion of a loop containing the two TMSs followed by a final maturation of the precursor protein by the inner membrane peptidase (IMP).

Calculations of mean hydrophobicity for the two TMSs of various COX2 and COX2A sequences were carried out as detailed in the Materials and methods. The aligned sequences of TMS1 and TMS2 are shown in Fig. S1, panels A and B, respectively. We compared the mean hydrophobicity of each TMS of COX2 proteins from green algae that are either mitochondria- or nucleus-encoded (Table S1). For comparative purposes, we also included the mitochondria- and the nucleus-encoded COX2 subunits from soybean [53], and the double mutant (L47Q/L49G) of soybean mitochondrial COX2 subunit engineered for cytosolic expression [36]. In addition, the mitochondria-encoded COX2 subunit of yeast [54] and its corresponding mutant (W56R) that can be expressed in the cytosol [37] were also included. This analysis allowed us to conclude that in average, the first TMS of COX2 expressed in the nucleus (or in a cytosolic vector) diminish their mean hydrophobicity from 1.77 (+/−0.030) to 1.50 (+/−0.048) as compared to their mitochondrial-encoded counterparts. In contrast, the second TMS of COX2 proteins expressed in the nucleus (or in a cytosolic vector) increase their mean hydrophobicity from 1.83 (+/−0.049) to 2.03 (+/−0.115) (Fig. S1 panel C). These simple calculations are in accordance with the route of biogenesis for *Polytomella* sp. COX2A subunit depicted in Fig. 5: while the first TMS has a diminished mean hydrophobicity that allows it to be fully translocated into the matrix, the second TMS exhibits an increased mean hydrophobicity that allows Tim23 to insert it laterally into the inner membrane.

In contrast to COX2A, COX2B lacks a canonical, cleavable MTS [7]. We propose that COX2B enters directly through TOM into the IMS, although it remains to be ascertained if an import pathway involving oxidation-driven reactions at the mitochondrial disulfide relay through Mia40 and Erv1 is involved [27]. The small but consistent increase in COX2B import observed upon DTT treatment argues in favor of a Mia40-mediated mechanism. Paradoxically, a reducing agent like DTT or glutathione may increase the rate of substrate oxidation by Mia40 and the Mia40-dependent protein import into the IMS [27]. Also, the primary sequence of COX2B contains the motif CX_3C that along with the CX_9C signature is characteristic of the proteins that constitute substrates for the mitochondrial disulfide relay system [55,56].

We hypothesize that after binding to the IMS-located Mia40 receptor, COX2B interacts with the newly synthesized COX2A subunit to form the heterodimeric COX2 subunit. After assembly, the last step that gives rise to the mature, functional COX2 must be the addition of the covalently bound copper diatomic center associated to subunit COX2B [57,58]. Alternatively, the copper could be inserted beforehand, and only then COX2B would assemble into the cytochrome *c* oxidase complex.

The time courses and the efficiency of internalization of COX2A and COX2B into mitochondria were strikingly different. COX2A import occurred at relatively short times of incubation and did not seem to increase further with time. The same happened with ALDH, a polypeptide that is known to enter the organelle in a $\Delta\Psi$-dependent process [18]. A time course of respiratory control measurements suggests that isolated mitochondria from the colorless alga are able to sustain a $\Delta\Psi$ for only relatively short periods of time. The apparent low efficiency of COX2A import (around 6% of the precursor reaching a protease-resistant, mature form as judged by densitometric analysis), is an underestimation due to a differential content of methionines in the proteins (five in the COX2A precursor and only one in the mature subunit). Assuming uniform labeling, the mature COX2A protein would carry only 1/5 of the radiolabeled amino acid incorporated by the COX2A precursor. This correction raises the actual efficiency of COX2A import to close to 20%. By contrast, COX2B import was highly efficient, and its internalization increased as a function of time in a process that was clearly $\Delta\Psi$-independent.

This work provides new insights on the biogenesis of chlorophycean COX2A and COX2B subunits, and illustrates how these unique nucleus-encoded mitochondrial proteins are imported into mitochondria. These polypeptides follow two distinct internalization pathways in order to assemble and give rise to the functional, heterodimeric COX2 subunit of cytochrome *c* oxidase.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2012.02.038.

Acknowledgements

We dedicate this paper to Professor Walter Neupert (LMU, Germany), whose contributions to the field inspired this project some years ago. The authors are grateful to Dr. Marina Gavilanes-Ruiz (Facultad de Química, UNAM) for her help with the statistical analysis, to Dr. Henry Weiner (Purdue University, USA) for his kind gift of the ALDH clone, and to Oscar Ramírez-Salcedo and Dr. Ruy Pérez-Montfort (IFC-UNAM) for their support in the early stages of the project. This work was partially supported by grants from Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) 128110 and Dirección General de Asuntos del Personal Académico (DGAPA-UNAM, Mexico) IN203311 to DGH; CONACyT 79989 and DGAPA-UNAM IN217109 to SUC; and CONACyT 105631 and DGAPA-UNAM IN215810 to SF. AJS is a Biochemistry PhD student at UNAM (fellowship 221018 from CONACyT).

References

[1] D.U. Mick, T.D. Fox, P. Rehling, Inventory control: cytochrome *c* oxidase assembly regulates mitochondrial translation, Nat. Rev. Mol. Cell Biol. 12 (2011) 14–20.
[2] C. Vahrenholz, G. Riemen, E. Pratje, B. Dujon, G. Michaelis, Mitochondrial DNA of *Chlamydomonas reinhardtii*: the structure of the ends of the linear 15.8-kb genome suggests mechanisms for DNA replication, Curr. Genet. 24 (1993) 241–247.
[3] E.M. Denovan-Wright, A.M. Nedelcu, R.W. Lee, Complete sequence of the mitochondrial DNA of *Chlamydomonas eugametos*, Plant Mol. Biol. 36 (1998) 285–295.
[4] J. Fan, R.W. Lee, Mitochondrial genome of the colorless green alga *Polytomella parva*: two linear DNA molecules with homologous inverted repeat termini, Mol. Biol. Evol. 19 (2002) 999–1007.
[5] X. Pérez-Martínez, M. Vázquez-Acevedo, E. Tolkunova, S. Funes, M.G. Claros, E. Davidson, M.P. King, D. González-Halphen, Unusual location of a mitochondrial gene. Subunit III of cytochrome c oxidase is encoded in the nucleus of Chlamydomonad algae, J. Biol. Chem. 275 (2000) 30144–30152.
[6] K.I. Watanabe, T. Ohama, Regular spliceosomal introns are invasive in *Chlamydomonas reinhardtii*: 15 introns in the recently relocated mitochondrial *cox2* and *cox3* genes, J. Mol. Evol. 53 (2001) 333–339.
[7] X. Pérez-Martínez, A. Antaramian, M. Vázquez-Acevedo, S. Funes, E. Tolkunova, J. d'Alayer, M.G. Claros, E. Davidson, M.P. King, D. González-Halphen, Subunit II of cytochrome c oxidase in Chlamydomonad algae is a heterodimer encoded by two independent nuclear genes, J. Biol. Chem. 276 (2001) 11302–11309.
[8] X. Pérez-Martínez, S. Funes, E. Tolkunova, E. Davidson, M.P. King, D. González-Halphen, Structure of nuclear-localized cox3 genes in *Chlamydomonas reinhardtii* and in its colorless close relative *Polytomella* sp. Curr. Genet. 40 (2002) 399–404.
[9] S.S. Merchant, S.E. Prochnik, O. Vallon, E.H. Harris, S.J. Karpowicz, G.B. Witman, A. Terry, A. Salamov, L.K. Fritz-Laylin, L. Maréchal-Drouard, W.F. Marshall, L.H. Qu, D.R. Nelson, A.A. Sanderfoot, M.H. Spalding, V.V. Kapitonov, Q. Ren, P. Ferris, E. Lindquist,

H. Shapiro, S.M. Lucas, J. Grimwood, J. Schmutz, P. Cardol, H. Cerutti, G. Chanfreau, C.L. Chen, V. Cognat, M.T. Croft, R. Dent, S. Dutcher, E. Fernández, H. Fukuzawa, D. González-Ballester, D. González-Halphen, A. Hallmann, M. Hanikenne, M. Hippler, W. Inwood, K. Jabbari, M. Kalanon, R. Kuras, P.A. Lefebvre, S.D. Lemaire, A.V. Lobanov, M. Lohr, A. Manuell, I. Meier, L. Mets, M. Mittag, T. Mittelmeier, J.V. Moroney, J. Moseley, C. Napoli, A.M. Nedelcu, K. Niyogi, S.V. Novoselov, I.T. Paulsen, G. Pazour, S. Purton, J.P. Ral, D.M. Riaño-Pachón, W. Riekhof, L. Rymarquis, M. Schroda, D. Stern, J. Umen, R. Willows, N. Wilson, S.L. Zimmer, J. Allmer, J. Balk, K. Bisova, C.J. Chen, M. Elias, K. Gendler, C. Hauser, M.R. Lamb, H. Ledford, J.C. Long, J. Minagawa, M.D. Page, J. Pan, W. Pootakham, S. Roje, A. Rose, E. Stahlberg, A.M. Terauchi, P. Yang, S. Ball, C. Bowler, C.L. Dieckmann, V.N. Gladyshev, P. Green, R. Jorgensen, S. Mayfield, B. Mueller-Roeber, S. Rajamani, R.T. Sayre, P. Brokstein, I. Dubchak, D. Goodstein, L. Hornick, Y.W. Huang, J. Jhaveri, Y. Luo, D. Martínez, W.C. Ngau, B. Otillar, A. Poliakov, A. Porter, L. Szajkowski, G. Werner, K. Zhou, I.V. Grigoriev, D.S. Rokhsar, A.R. Grossman, The Chlamydomonas genome reveals the evolution of key animal and plant functions, Science 318 (2007) 245–250.

[10] S.E. Prochnik, J. Umen, A.M. Nedelcu, A. Hallmann, S.M. Miller, I. Nishii, P. Ferris, A. Kuo, T. Mitros, L.K. Fritz-Laylin, U. Hellsten, J. Chapman, O. Simakov, S.A. Rensing, A. Terry, J. Pangilinan, V. Kapitonov, J. Jurka, A. Salamov, H. Shapiro, J. Schmutz, J. Grimwood, E. Lindquist, S. Lucas, I.V. Grigoriev, R. Schmitt, D. Kirk, D.S. Rokhsar, Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*, Science 329 (2010) 223–226.

[11] R. van Lis, D. González-Halphen, A. Atteia, Divergence of the mitochondrial electron transport chains from the green alga *Chlamydomonas reinhardtii* and its colorless close relative *Polytomella* sp. Biochim. Biophys. Acta 1708 (2005) 23–34.

[12] A. Atteia, R. van Lis, S.I. Beale, Enzymes of the heme biosynthetic pathway in the nonphotosynthetic alga *Polytomella* sp. Eukaryot. Cell 4 (2005) 2087–2097.

[13] G.H. Hogeboom, W.C. Schneider, G.E. Pallade, Cytochemical studies of mammalian tissues; isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material, J. Biol. Chem. 172 (1948) 619–635.

[14] P. Cavadani, O. Galkn, G. Isaya, Protein import and processing reconstituted with isolated rat liver mitochondria and recombinant mitochondrial processing peptidase, Methods 26 (2002) 298–306.

[15] A.G. Gornall, B.C. Jardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–766.

[16] J.M. Herrmann, H. Fölsch, W. Neupert, R.A. Stuart, Isolation of yeast mitochondria and study of mitochondrial protein translation, in: J.E. Celis (Ed.), Cell Biology A Laboratory Handbook, vol. 1, Academic Press, San Diego, 1994, pp. 538–544.

[17] B. Guérin, P. Labbe, M. Somlo, Preparation of yeast mitochondria (*Saccharomyces cerevisiae*) with good P/O and respiratory control ratios, Methods Enzymol. 55 (1979) 149–159.

[18] P.K. Hammen, M. Waltner, B. Hahnemann, T.S. Heard, H. Weiner, The role of positive charges and structural segments in the presequence of rat liver aldehyde dehydrogenase in import into mitochondria, J. Biol. Chem. 271 (1996) 21041–21048.

[19] J.M. Scholt, D. Barrick, E.J. York, J.M. Stewart, R.L. Balkwin, Urea unfolding of peptide helices as a model for interpreting protein unfolding, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 185–189.

[20] H. Schägger, Native gel electrophoresis, in: G. von Jagow, H. Schägger (Eds.), A Practical Guide to Membrane Protein Purification, Academic Press, San Diego, 1994, pp. 81–104.

[21] J.M. Herrmann, W. Neupert, R.A. Stuart, Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear-encoded Oxa1p, EMBO J. 16 (1997) 2217–2226.

[22] H. Bauerschmitt, S. Funes, J.M. Herrmann, Synthesis and sorting of mitochondrial translation products, Methods Mol. Biol. 457 (2008) 95–112.

[23] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[24] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.

[25] B. Schmidt, E. Wachter, W. Sebald, W. Neupert, Processing peptidase of Neurospora mitochondria. Two-step cleavage of imported ATPase subunit 9, Eur. J. Biochem. 144 (1984) 581–588.

[26] Y. Fujiki, S. Fowler, H. Shio, A.L. Hubbard, P.B. Lazarow, Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes, J. Cell Biol. 93 (1982) 103–110.

[27] J.M. Herrmann, J. Riemer, The mitochondrial disulfide relay: redox-regulated protein import into the intermembrane space, J. Biol. Chem. (Dec 6 2011) [Epub ahead of print].

[28] G.H. Clarkson, R.O. Poyton, A role for membrane potential in the biogenesis of cytochrome c oxidase subunit II, a mitochondrial gene product, J. Biol. Chem. 264 (1989) 10114–10118.

[29] J.M. Herrmann, N. Bonnefoy, Protein export across the inner membrane of mitochondria: the nature of translocated domains determines the dependence on the Oxa1 translocase, J. Biol. Chem. 279 (2004) 2507–2512.

[30] H.L. Fiumera, S.A. Broadley, T.D. Fox, Translocation of mitochondrially synthesized Cox2 domains from the matrix to the intermembrane space, Mol. Cell. Biol. 27 (2007) 4664–4673.

[31] K.A. Sevarino, R.O. Poyton, Mitochondrial membrane biogenesis: identification of a precursor to yeast cytochrome c oxidase subunit II, an integral polypeptide, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 142–146.

[32] E. Pratje, G. Mannhaupt, G. Michaelis, K. Beyreuther, A nuclear mutation prevents processing of a mitochondrially encoded membrane protein in *Saccharomyces cerevisiae*, EMBO J. 2 (1983) 1049–1054.

[33] A.T. Torello, M.H. Overholtzer, V.L. Cameron, N. Bonnefoy, T.D. Fox, Deletion of the leader peptide of the mitochondrially encoded precursor of *Saccharomyces cerevisiae* cytochrome *c* oxidase subunit II, Genetics 145 (1997) 903–910.

[34] K.L. Adams, K. Song, P.G. Roessler, J.M. Nugent, J.L. Doyle, J.J. Doyle, J.D. Palmer, Intracellular gene transfer in action: dual transcription and multiple silencings of nuclear and mitochondrial cox2 genes in legumes, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 13863–13868.

[35] K.L. Adams, J.D. Palmer, Evolution of mitochondrial gene content: gene loss and transfer to the nucleus, Mol. Phylogenet. Evol. 29 (2003) 380–395.

[36] D.O. Daley, R. Clifton, J. Whelan, Intracellular gene transfer: reduced hydrophobicity facilitates gene transfer for subunit 2 of cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 10510–10515.

[37] S. Funes, E. Davidson, M.G. Claros, R. van Lis, X. Pérez-Martinez, M. Vázquez-Acevedo, M.P. King, D. González-Halphen, The typically mitochondrial DNA-encoded ATP6 subunit of the F1F0-ATPase is encoded by a nuclear gene in *Chlamydomonas reinhardtii*, J. Biol. Chem. 277 (2002) 6051–6058.

[38] L. Supekova, F. Supek, J.E. Greer, P.G. Schultz, A single mutation in the first transmembrane domain of yeast COX2 enables its allotopic expression, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 5047–5052.

[39] S. Funes, E. Davidson, A. Reyes-Prieto, S. Magallón, P. Herion, M.P. King, D. González-Halphen, A green algal apicoplast ancestor, Science 298 (2002) 2155.

[40] G. Nurani, M. Eriksson, C. Knorpp, E. Glaser, L.-G. Franzén, Homologous and heterologous protein import into mitochondria isolated from the green alga *Chlamydomonas reinhardtii*, Plant Mol. Biol. 35 (1997) 973–980.

[41] S. Funes, L.-G. Franzén, D. González-Halphen, Chlamydomonas reinhardtii: the model of choice to study mitochondria from unicellular photosynthetic organisms, Methods Mol. Biol. 372 (2007) 137–149.

[42] P. Rustin, B. Parfait, D. Chretien, T. Bourgeron, F. Djouadi, J. Bastin, A. Rotig, A. Munnich, Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells, J. Biol. Chem. 271 (1996) 14785–14790.

[43] S. Todisco, G. Agrimi, A. Castegna, F. Palmieri, Identification of the mitochondrial NAD^+ transporter in *Saccharomyces cerevisiae*, J. Biol. Chem. 281 (2006) 1524–1531.

[44] M. Neuburger, R. Douce, Slow passive diffusion of NAD^+ between intact isolated plant mitochondria and suspending medium, Biochem. J. 216 (1983) 443–450.

[45] F. Figueroa-Martínez, S. Funes, L.-G. Franzén, D. González-Halphen, Reconstructing the mitochondrial protein import machinery of *Chlamydomonas reinhardtii*, Genetics 179 (2008) 149–155.

[46] C. Carrie, M.W. Murcha, J. Whelan, An *in silico* analysis of the mitochondrial protein import apparatus of plants, BMC Plant Biol. 10 (2010) 249.

[47] D. Mokranjac, W. Neupert, Thirty years of protein translocation into mitochondria: unexpectedly complex and still puzzling, Biochim. Biophys. Acta 1793 (2009) 33–41.

[48] S. Kutik, D.A. Stroud, N. Wiedemann, N. Pfanner, Evolution of mitochondrial protein biogenesis, Biochim. Biophys. Acta 1790 (2009) 409–415.

[49] D.O. Daley, K.L. Adams, R. Clifton, S. Qualmann, A.H. Millar, J.D. Palmer, E. Pratje, J. Whelan, Gene transfer from mitochondrion to nucleus: novel mechanisms for gene activation from Cox2, Plant J. 30 (2002) 11–21.

[50] D. González-Halphen, S. Funes, X. Pérez-Martínez, A. Reyes-Prieto, M.G. Claros, E. Davidson, M.P. King, Genetic correction of mitochondrial diseases: using the natural migration of mitochondrial genes to the nucleus in chlorophyte algae as a model system, Ann. N. Y. Acad. Sci. 1019 (2004) 232–239.

[51] M. Kübrich, J. Rassow, W. Voos, N. Pfanner, A. Hönlinger, The import route of ADP/ATP carrier into mitochondria separates from the general import pathway of cleavable preproteins at the trans side of the outer membrane, J. Biol. Chem. 273 (1998) 16374–16381.

[52] P. Rehling, K. Model, K. Brandner, P. Kovermann, A. Sickmann, H.E. Meyer, W. Kühlbrandt, R. Wagner, K.N. Truscott, N. Pfanner, Protein insertion into the mitochondrial inner membrane by a twin-pore translocase, Science 299 (2003) 1747–1751.

[53] P.S. Covello, M.W. Gray, Silent mitochondrial and active nuclear genes for subunit 2 of cytochrome c oxidase (cox2) in soybean: evidence for RNA-mediated gene transfer, EMBO J. 11 (1992) 3815–3820.

[54] F. Foury, T. Roganti, N. Lecrenier, B. Purnelle, The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*, FEBS Lett. 440 (1998) 325–331.

[55] F. Arnesano, E. Balatri, L. Banci, I. Bertini, D.R. Winge, Folding studies of Cox17 reveal an important interplay of cysteine oxidation and copper binding, Structure 13 (2005) 713–722.

[56] C.T. Webb, M.A. Gorman, M. Lazarou, M.T. Ryan, J.M. Gulbis, Crystal structure of the mitochondrial chaperone TIM9.10 reveals a six-bladed alpha-propeller, Mol. Cell 21 (2006) 123–133.

[57] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A, Science 272 (1996) 1136–1144.

[58] K. Rigby, P.A. Cobine, O. Khalimonchuk, D.R. Winge, Mapping the functional interaction of Sco1 and Cox2 in cytochrome oxidase biogenesis, J. Biol. Chem. 283 (2008) 15015–15022.