The polypeptides COX2A and COX2B are essential components of the mitochondrial cytochrome *c* oxidase of *Toxoplasma gondii*

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Abstract

Two genes encoding cytochrome *c* oxidase subunits, *Cox2a* and *Cox2b*, are present in the nuclear genomes of apicomplexan parasites and show sequence similarity to corresponding genes in chlorophycean algae. We explored the presence of COX2A and COX2B subunits in the cytochrome *c* oxidase of *Toxoplasma gondii*. Antibodies were raised against a synthetic peptide containing a 14-residue fragment of the COX2A polypeptide and against a hexa-histidine-tagged recombinant COX2B protein. Two distinct immunochemical stainings localized the COX2A and COX2B proteins in the parasite's mitochondria. A mitochondria-enriched fraction exhibited cyanide-sensitive oxygen uptake in the presence of succinate. *T. gondii* mitochondria were solubilized and subjected to Blue Native Electrophoresis followed by second dimension electrophoresis. Selected protein spots from the 2D gels were subjected to mass spectrometry analysis and polypeptides of mitochondrial complexes III, IV and V were identified. Subunits COX2A and COX2B were detected immunochemically and found to co-migrate with complex IV; therefore, they are subunits of the parasite's cytochrome *c* oxidase. The apparent molecular mass of the *T. gondii* mature COX2A subunit differs from that of the chlorophycean alga *Polytomella* sp. The data suggest that during its biogenesis, the mitochondrial targeting sequence of the apicomplexan COX2A precursor protein may be processed differently than the one from its algal counterpart.
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1. Introduction

Mitochondrial cytochrome *c* oxidase (EC 1.9.3.1), or complex IV, the terminal component of the respiratory chain, transfers electrons from reduced cytochrome *c* to oxygen. This reaction is coupled to proton translocation from the matrix to the intermembrane space [1,2]. Cytochrome *c* oxidase is an oligomeric membrane protein complex of 10–13 subunits that contains four redox centers: a binuclear Cu_A, heme *a*, heme a_3 and Cu_B [3]. In the majority of eukaryotes, the three largest subunits of cytochrome *c* oxidase, COX1, COX2 and COX3, are encoded by the corresponding *cox1*, *cox2* and *cox3* genes of the mitochondrial genome [4]. In chlorophycean algae, i.e., *Chlamydomonas reinhardtii*, *Volvox carterii* and *Polytomella* sp., the *cox2* gene is fragmented in two distinct genes, *Cox2a* and *Cox2b*, located in different chromosomes of the nuclear genome [5–8]. The *Cox2a* gene encodes subunit COX2A, homologous to the amino terminal half of a typical one-polypeptide COX2, while the *Cox2b* gene encodes subunit COX2B, equivalent to the C-terminal half of an orthodox COX2. Fragmented COX2 subunits contain unique amino acid extensions: a C-terminal extension in COX2A and a N-terminal extension in COX2B, which are thought to stabilize the interaction between both polypeptides [5]. In addition, the COX2A precursor protein

contains a long mitochondrial targeting sequence (MTS) of 130 residues [5]. The non-covalent assembly of the COX2A and COX2B subunits in the inner mitochondrial membrane gives rise to a heterodimeric COX2 subunit in green algal cytochrome *c* oxidase. *Cox2a* and *Cox2b* genes are also present in the nuclear genome of two taxa of alveolates (protists with a layer of flattened vesicles): apicomplexan parasites (named for their distinctly polarized cell apex) [7,9] and dinoflagellates (flagellated, often photosynthetic protists) [10]. These findings correlate with the fact that all mitochondrial genomes of apicomplexan parasites characterized to date contain only the cytochrome oxidase encoding genes *cox1* and *cox3*, while the *cox2* gene is invariably absent [11]. The presence of COX2A and COX2B subunits in the cytochrome *c* oxidase of chlorophycean has been well documented [5,12,13], but the presence of equivalent subunits in apicomplexan parasites has not been ascertained. Here, we explore the presence of COX2A and COX2B subunits in the mitochondrion of the apicomplexan parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis. Using different microscopy techniques, we immunolocalized the COX2A and COX2B subunits in *T. gondii* mitochondria. We also describe the isolation of a mitochondria-enriched fraction from the parasite and the resolution of its protein components by Blue Native Electrophoresis (BNE), and provide immunochemical evidence for the association of COX2A and COX2B subunits with *T. gondii* cytochrome *c* oxidase. The data also suggest that the apicomplexan parasite COX2A precursor protein may be processed differently during its biogenesis as compared to its chlorophycean algal counterpart.

2. Materials and methods

2.1. Strains and cultures

T. gondii tachyzoites were cultured as previously reported [14]. Tachyzoites are the stage of the parasite that rapidly multiplies in any cell of the intermediate host and in nonintestinal epithelial cells of the definitive host. Nine-week old female mice (strain Balb/c) were inoculated intraperitoneally with 1×10^6 *T. gondii* tachyzoites strain RH in sterile PBS (pH 7.2). The parasites were recovered four days post-infection from peritoneal exudates. Exudates were passed through needles of different calibers to eliminate contaminating cells, and centrifuged at 1200 ×*g* for 10 min. The supernatant was filtered through a polycarbonate filter (pore diameter of 5 µm) and centrifuged again at 2400 ×*g* for 10 min. Freshly harvested tachyzoites were used for microscopy studies. Otherwise, they were resuspended in a small volume of PBS and stored at −70 °C until used.

2.2. Transmission electron microscopy

T. gondii tachyzoites (10×10^6 cells) were fixed with 2.5 glutaraldehyde in PBS (pH 7.2) for 2 h at 4 °C, and washed three times with PBS by centrifugation at 2400 ×*g* in a table-top centrifuge. The parasite cells were post-fixed with 1% osmium tetroxide. Dehydration was carried out at room temperature in a graded series of ethanol at a concentration from 40 to 100% (v/v) in 10% increments. Then, samples were placed two times during 15 min each in propylene oxide. Pre-embedding in 1:1 propylene oxide-epoxy resin was conducted overnight. Thin sections (50 to 60 nm thick) were cut with an ultramicrotome (Leica Ultracut R) and placed onto formvar-coated 200 mesh copper grids. Grids were contrasted with uranyl acetate and lead citrate and examined under a JEOL 1200 EX II transmission electron microscope operating at 80 kV.

2.3. Antibody production against COX2A subunit

The sequence of *T. gondii* COX2A subunit (accession numbers AAO27883 and AF503914) [15] was analyzed for possible antigenic sites at the Molecular Immunology Foundation Homepage (http://bio.dfci.harvard.edu/Tools/antigenic.html) following the semi-empirical method for prediction of antigenic determinants of Kolaskar and Tongaonkar [16]. The 14-residue antigenic determinant ETRDLVELFLVHQK was chosen. An extra cysteine was added to the N-terminus of this peptide to allow crosslinking with maleimidobenzoyl-*N*-hydroxysuccinimide ester to a protein carrier for antibody production. Peptide synthesis and coupling to keyhole limpet hemocyanin was carried out by NeoMPS (San Diego, CA). The coupled peptide was then used to raise antibodies in rabbits.

2.4. COX2B overexpression and antibody production

Primers were designed based on the sequence of the *Cox2b* gene (accession numbers AAO27882 and AF499004) [15]: 5′-CGC GGA TCC GAT GTT TTC CGG TCC TTC TGG AAC-3′ and 5′-CGG GGT ACC TCA GTC TTT GTA CCA CTT CTT TGC-3′. Underlined are the added *BamHI* and *KpnI* restriction sites included to facilitate cloning. A cDNA library of *T. gondii* was the template for PCR amplification with *Taq* polymerase [15]. The 560-bp product was cloned into the restriction sites *BamHI* and *KpnI* of the pQE30 vector (Qiagen) and the COX2B protein of 23.8 kDa containing a six-residue histidine tag at the N-terminus was overexpressed in an *Escherichia coli* XL1 blue strain in the presence of 1 mM IPTG (37 °C for 4 h). The overexpressed polypeptide, which was mainly found in inclusion bodies, was extracted in the presence of 6 M guanidine–HCl, purified using a Ni-NTA agarose resin, and used to raise anti-COX2B antibodies in a rabbit.

2.5. Immunoelectron microscopy

The method described by Agredano-Moreno, et al. [17] was adapted for *T. gondii* cells. Tachyzoites (10×10^6 cells) were fixed with 4% paraformaldehyde followed by dehydration with increasing concentrations of methanol, and embedded in Lowicryl K4M resin. Polymerization was carried out in Eppendorf tubes for 55 min under UV light (320–360 nm). Thin sections (60 nm thick) were placed onto 200-mesh formvar-coated gold-grids and incubated for 2 h with 0.1% TBST (pH 7.6) in a humid chamber at room temperature. The grids were washed three times with TBS (pH 7.5) and incubated with anti-COX2B antibody (1:50 dilution in TBS) overnight at 4 °C in the humid chamber, washed four times with TBS, and blocked with 1% ovoalbumin in 0.01 M PBS for 30 min as described [18]. Grids were then incubated with the secondary antibody (goat anti-rabbit IgG coupled to gold beads of 12 nm from Jackson ImmunoResearch) in a 1:20 dilution. Subsequently, the grids were washed four times with TBS and five times with distilled water. Finally, they were contrasted with uranyl acetate and lead citrate and observed under a JEOL JEM 1010 electron microscope coupled to a digital camera MTi model CCD-300-RC.

2.6. Mitotracker staining of T. gondii tachyzoites

A suspension of living parasites (100 µl, containing 12.5×10^6 tachyzoites) was incubated with 2.5 µM Mitotracker CMX Ros (Molecular Probes) at room temperature and in the dark for 20 min. The cells were washed four times with PBS, resuspended in 10 µl of the same buffer, and loaded on slides covered with 0.1 M poly-L-lysine. The samples were cover-slipped and observed under a Laser Scanning Confocal Microscope Fluoview FV 1000 Spectral (Olympus) using a helium–neon laser emitting at 543 nm. Where indicated, tachyzoites were also incubated in the presence of DAPI (1 µg/ml) to stain nuclei, and blue fluorescence was followed with a laser diode emitting at 405 nm. Also, where indicated, before labeling with Mitotracker, 6.25×10^4 tachyzoites were incubated in the presence of either 1 mM NaCN, 250 µM Antimycin A, or 6 mM CCCP during 30 min at room temperature.

2.7. Confocal immunolocalization studies

T. gondii tachyzoites (6.25×10^4) were labeled with 2.5 µM Mitotracker CMX Ros (Molecular Probes) at room temperature and in the dark for 30 min.

The cells were then washed four times by centrifugation (2400 ×*g* for 10 min) with PBS pH 7.2. The parasite cells were permeabilized with 0.05% Tween 20 for 90 min at room temperature, washed four times, and blocked with 1% BSA for 1 h as previously described [19]. The tachyzoites were incubated at 4 °C overnight either with the rabbit anti-Toxoplasma COX2B antibody (1:10 dilution) or with the rabbit anti-Toxoplasma COX2A antibody (1:5 and 1:10 dilutions), washed three times with PBS and incubated for 2 h with fluorochrome-conjugated secondary antibodies (goat anti-rabbit Alexa Fluor® 488 from Molecular Probes, 1:100 dilution in PBS containing 1% BSA). Primary antibodies were omitted from samples used as negative controls. The cells were washed three times with PBS and incubated with DAPI (1 µg/ml) for 30 min at room temperature, and washed again four times. The cells were fixed with 4% paraformaldehyde in PBS and washed three times. Finally, the tachyzoites were pelleted by centrifugation at 3600 ×*g* for 10 min and resuspended in 10 µl of PBS and observed under the Laser Scanning Confocal Microscope. Alexa 488 (green fluorescence) was followed with an argon laser emitting at 488 nm.

2.8. Isolation of mitochondria and oxygen uptake measurements

For the isolation of the mitochondrial fraction, we modified the protocol of Leriche and Dubremetz [20], originally developed for the isolation of *T. gondii* secretory organelles. All steps were carried out at 4 °C. *T. gondii* tachyzoites (250×10^6) were thawed and washed twice by centrifugation at 2000 ×*g* for 10 min and resuspension in 10 ml of a buffer containing 250 mM sucrose, 1 mM potassium EDTA, and 5 mM triethanolamine (pH 7.5) (TES buffer). The pellet was resuspended in 10 ml of TES buffer and broken mechanically with a Potter homogenizer with five to six gentle manual strokes of the teflon-pestle. The broken cells were centrifuged at 2000 ×*g* for 10 min and the supernatant centrifuged at 27,000 ×*g* for 30 min. The resulting pellet was resuspended in a small volume of TES buffer, taken to a final concentration of 40% Percoll, and loaded onto 10 ml centrifuge tubes containing 40% Percoll in TES buffer. Samples were centrifuged at 180,000 ×*g* for 45 min in a 50Ti rotor. Fractions of 700 µl each were collected, and the mitochondrial fraction identified by measuring the oxygen uptake of each fraction in the presence of 10 mM succinate at 27 °C. A Clark-oxygen electrode in a reaction vessel of 3 ml of air-saturated respiration buffer (5 mM MES, 0.6 M mannitol, 4 mM H_2PO_4, 20 mM KCl, and 0.5 mM $MgCl_2$ at pH 6.8) was used. Measurements were performed with 0.25–0.35 mg/ml of protein of the mitochondrial-enriched fraction.

Table 1
Species that contain fragmented *Cox2a* and *Cox2b* genes

| Lineage	Species	COX2A	COX2B
Dinoflagellata	*Alexandrium tamarense*		CK782370
	Karenia brevis		CO060735
	Karlodinium micrum	ABF22753.1	KME00016886
	Oxyrrhis marina	ABF22755.1	ABF22756.1
Apicomplexa	*Eimeria tenella*	CD666153	
	Neospora caninum	CF423061	CF275235.1
	Plasmodium berghei	XP_680277.1	XP_678991.1
	Plasmodium chabaudi	XP_738170.1	XP_731807.1
	Plasmodium falciparum	XP_001350328.1	XP_001348462.1
	Plasmodium yoelii	EAA20212.1	XP_728989.1
	Sarcocystis falcatula		DV180913
	Theileria annulata	XP_954616.1	
	Theileria parva	XP_765620.1	XP_766204.1
	Toxoplasma gondii	AAO27883.1	AAO27882.1
Chlorophyceae	*Chlamydomonas reinhardtii*	AF443624_1	AF305543_1
	Haematococcus pluvialis	DV203769	DV203283
	Polytomella parva	POE00000962	POE00004826
	Polytomella sp.	AF305541_1	AF305542_1
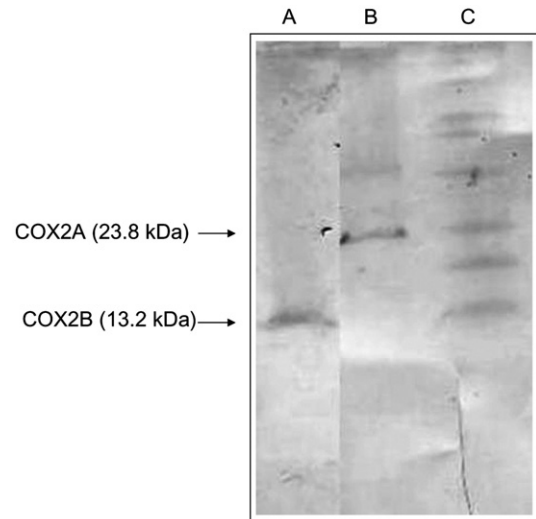
Fig. 1. Immunoblot analysis of whole *T. gondii* cell extracts. *T. gondii* whole cell protein extracts (equivalent to 40 µg of protein) were subjected to denaturing SDS-PAGE and transferred to nylon membranes for Western blot analyses). A) Lane decorated with the anti-COX2B antibody. B) Lane decorated with the anti-COX2A antibody. C) Molecular mass markers. Two immunoreactive bands with apparent molecular masses of 23.8 kDa (COX2A) and 13.2 kDa (COX2B) could be identified.

2.9. Blue native polyacrylamide gel electrophoresis

Sample preparation and BN-PAGE were carried out as described by Schägger and von Jagow [21]. Mitochondria were thawed and centrifuged at maximum velocity of a table-top centrifuge for 5 min. The pellet was resuspended in TES buffer, centrifuged again and the supernatant was discarded. Mitochondria were then resuspended in sample buffer (50 mM Bis–Tris, 0.75 M amino caproic acid, pH 7.0) to a final protein concentration of 5 mg/ml and solubilized in the presence of 2% (w/v) *n*-dodecyl maltoside at the same protein concentration for 30 min at 4 °C. After centrifugation at 100,000 ×*g* for 30 min, Coomassie Blue was added and the sample loaded onto 5–15% (w/v) gradient polyacrylamide gels.

2.10. 2D-SDS-PAGE

Entire lanes from BN-PAGE were used to resolve the subunits in the second dimension Tricine-SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE) (15% acrylamide) as previously described [21]. Apparent molecular masses were estimated using BenchMark protein standards (Invitrogen).

2.11. Protein analysis

Protein concentrations were determined as described [22]. After electrophoresis, proteins were electrotransferred onto nitrocellulose (BioRad) for Western blotting. The antisera used were raised against *T. gondii* COX2A and COX2B subunits (see below). For protein identification, Coomassie Blue-stained spots were excised from the gel and sent to the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, USA) for Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC–MS/MS) analysis.

2.12. Sequence analysis in silico

Protein sequences were obtained from ENTREZ at the NCBI server (www.ncbi.nlm.nih.gov). Multiple alignments were done with Clustal W (searchlauncher.bcm.tmc.edu). Molecular masses and p*I* calculations were done with the compute p*I*/MW tool [23] at the ExPASy Molecular Biology Server (www.expasy.ch).
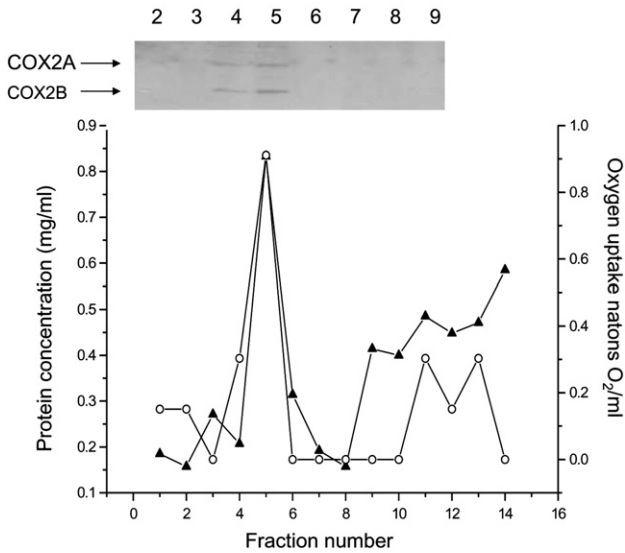
Fig. 2. Subcellular fractionation of *T. gondii* tachyzoites by Percoll gradient centrifugation. Bottom panel, elution profile of the Percoll gradients. Protein concentration (black triangles) and oxygen consumption rates (open circles) for each fraction are shown. Upper panel, immunoblot analysis of selected fractions forms the Percoll gradient. Numbers on top indicate the corresponding fraction number. Fractions exhibiting anti-COX2A and anti-COX2B immunoreactivity were those that exhibited maximal oxygen consumption activity using succinate as substrate.

3. Results

Nucleus-localized *Cox2a* and *Cox2b* genes are present in a limited number of eukaryotic lineages: chlorophycean algae, apicomplexan parasites, and in some dinoflagellates. Table 1 summarizes the COX2A and COX2B sequences that have been identified to date and the corresponding accession numbers. Although *Cox2a* and *Cox2b* genes are present in several apicomplexan parasites, until now, biochemical evidence for the presence of the corresponding protein products is lacking. This work explored the presence of mature COX2A and COX2B subunits assembled in the cytochrome *c* oxidase complex of an apicomplexan parasite. We generated specific antibodies against the parasite's COX2A and COX2B polypeptides. Anti-COX2A antibodies were induced against a 15-residue synthetic peptide, while anti-COX2B antibodies were generated against the 6-His-tagged recombinant protein. *T. gondii* whole cell protein extracts were subjected to denaturing SDS-PAGE and transferred to nylon membranes for Western blot analyses. Two immunoreactive bands with apparent molecular masses of 23.8 kDa (COX2A) and 13.2 kDa (COX2B) could be identified (Fig. 1). In a parallel gel, using the same set of molecular mass markers, *Polytomella* sp. extracts were run and transferred to membranes for Western blotting, using antibodies raised against the algal COX2A and COX2B subunits. The immunoreactive bands of the alga exhibited apparent molecular masses of 13.0 kDa (COX2A) and 15.0 kDa (COX2B) (data not shown).

Western blot analyses were also carried out with a *T. gondii* cell fraction enriched in mitochondria. For this purpose,
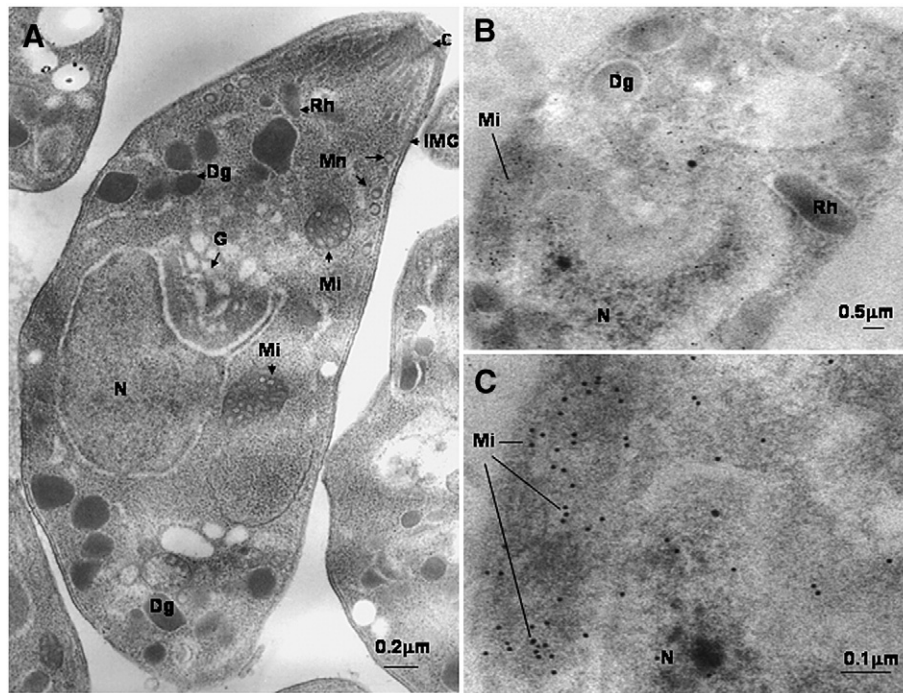

Fig. 3. Ultrastructure of a *T. gondii* taquizoite and immunolocalization of COX2B to mitochondria by electron microscopy using gold-labeled secondary antibodies. A) Electron micrograph of a sectioned tachyzoite. The main subcellular structures are indicated: conoids (C), dense granules (Dg), Golgi apparatus (G), internal membrane complex (IMC), rhoptries (Rh), micronemes (Mn), mitochondria (Mi) and nucleus (N). Immunolocalization of COX2B to *T. gondii* mitochondria (Mi). The other observed subcellular structures are also indicated: dense granule (Dg), rhoptries (Rh), nucleus (N). C) Higher magnification of panel B, that shows intense labeling in the mitochondrion.
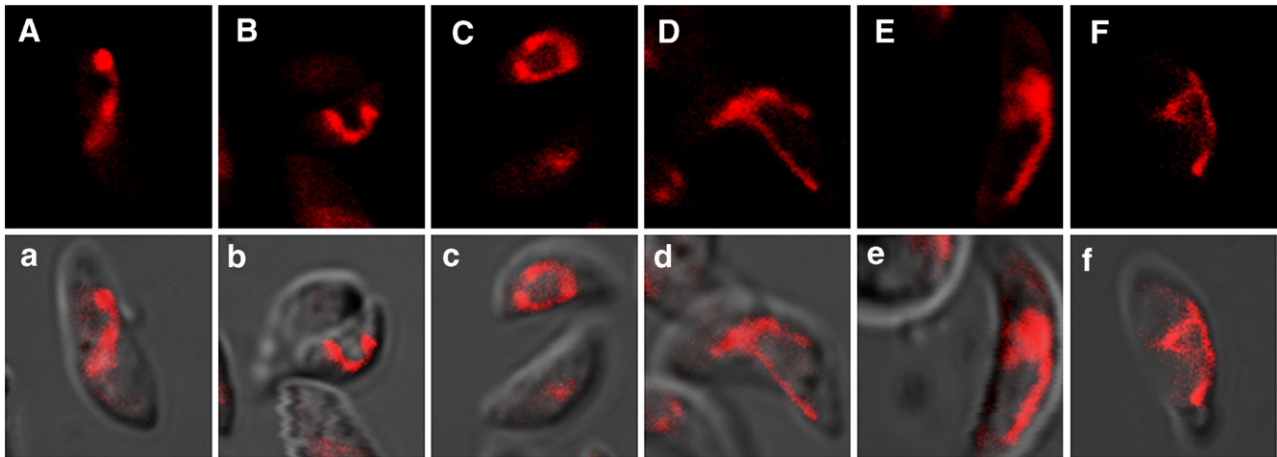
Fig. 4. Fluorescent images of mitochondria of *T. gondii* tachyzoites labeled with Mitotracker. Panels A, B, C, D, E and F show the different mitochondrial structures found in tachyzoites incubated in the presence of 2.5 μM Mitotracker Red CMX Ros. Panels a, b, c, d, e and f show the corresponding superimposed images of Mitotracker-stained mitochondria and tachyzoites observed under differential interference contrast.
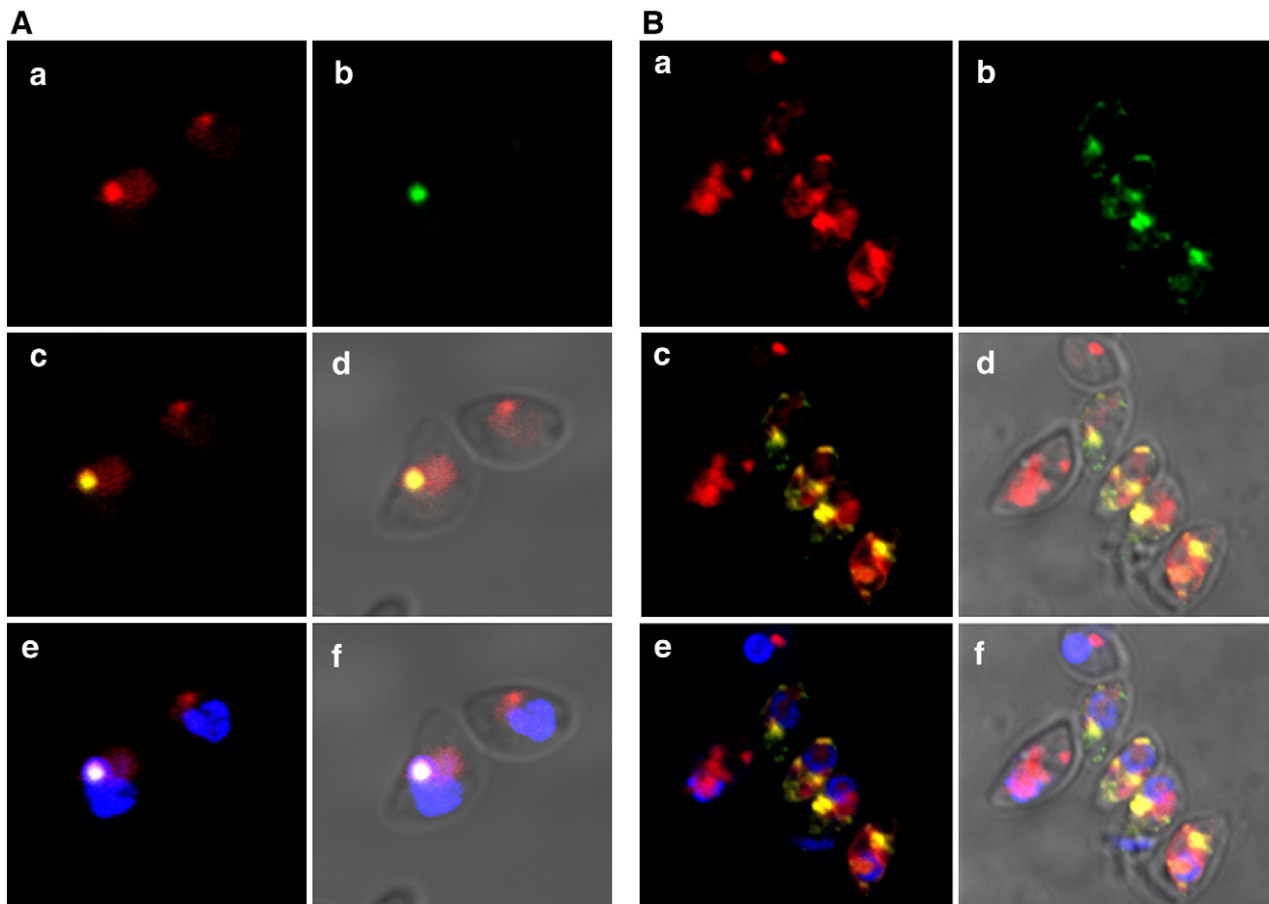

Fig. 5. Immunolocalization of COX2A and COX2B polypeptides by confocal microscopy of permeabilized tachyzoites using fluorochrome-labeled secondary antibodies. Panel A: a) Staining of mitochondria with Mitotracker (red fluorescence). b) Labeling with the rabbit anti-Toxoplasma COX2A antibody followed by the Alexa 488-conjugated anti-rabbit antibodies (green fluorescence). c) Colocalization of antibody fluorescence and Mitotracker staining. d) Overlay of panel "c" and tachyzoites observed under differential interference contrast. e) Staining of the nucleus with DAPI (blue fluorescence) and of mitochondria with Mitotracker (red fluorescence). f) Overlay of panel "e" and tachyzoites observed under differential interference contrast. Panel B: a) Staining of mitochondria with Mitotracker (red fluorescence). b) Labeling with the rabbit anti-Toxoplasma COX2B antibody followed by the Alexa 488-conjugated anti-rabbit antibodies (green fluorescence). c) Colocalization of antibody fluorescence and Mitotracker staining. d) Overlay of panel "c" and tachyzoites observed under differential interference contrast. e) Triple overlay of stainings: nucleus with DAPI (blue fluorescence), mitochondria with Mitotracker (red fluorescence), and anti-Toxoplasma COX2B antibodies (green fluorescence). f) Overlay of panel "e" and tachyzoites observed under differential interference contrast.

ruptured *T. gondii* tachyzoites were fractionated by ultracentrifugation on Percoll gradients. Cyanide-sensitive oxygen uptake in the presence of succinate was detected in several fractions of the Percoll gradient indicating the presence of mitochondria (Fig. 2). The antisera raised against the *T. gondii* COX2A and COX2B subunits were used to probe the polypeptides in the Percoll gradient fractions. As shown in Fig. 2, the cross-reactive polypeptides were found only in the fractions exhibiting maximal oxygen consumption, indicating that the two immunoreactive bands that correspond to the COX2A and COX2B polypeptides are associated to mitochondria. The same set of antibodies were then used to immunolocalize COX2A and COX2B polypeptides in *T. gondii*. Two experimental approaches were followed: immunolocalization by electron microscopy using gold-labeled secondary antibodies and immunodecoration by confocal microscopy of permeabilized tachyzoites using fluorochrome-labeled secondary antibodies. *T. gondii* possesses one or few branched mitochondria, with variations in shape and substructural organization [24–27]. The mitochondrial structures were readily identified by electron microscopy in sectioned tachyzoites (Fig. 3A). Although the Epon-embedded samples reveal a detailed fine-structure of the mitochondria cristae, Lowicryl K4M resin-embedded samples did not (Fig. 3B and C as compared to A). Nevertheless, Lowicryl K4M was the only resin that allowed immunogold localization. Immunogold electron microscopy of Lowicryl K4M resin-embedded tachyzoites decorated with anti-COX2B antibodies show high density

labeling associated with mitochondria (6.5 gold particles/μm^2 in mitochondria against 0.7 gold particles/μm^2 in the rest of the cell) (Fig. 3B and C).

The localization of COX2B in *T. gondii* tachyzoites was also examined by confocal immunofluorescence analysis. To illuminate mitochondria, living parasites were stained with Mitotracker (red fluorescence, Fig. 4). This lipophilic cationic dye is able to accumulate only in the matrix of mitochondria that have built up a membrane potential. If *T. gondii* cells were preincubated with either cyanide, antimycin or CCCP, Mitotracker was not taken up by the organelle, and diffused throughout the parasite's cytoplasm (results not shown). The confocal images of Mitotracker-stained *T. gondii* mitochondria were similar to those previously obtained [27], revealing the unusual structural features of an ovoid mitochondrial cytoplasmic complex. Mitochondria-internalized Mitotracker links covalently to matrix proteins, preventing its wash-out after cell permeabilization. Thus, tachyzoites containing Mitotracker-stained mitochondria were permeabilized with low concentrations of detergent and incubated with anti-COX2A and anti-COX2B antibodies, followed by incubation with fluorochrome-labeled secondary antibodies. The immunolocalization of COX2A and COX2B (green fluorescence, Fig. 5) was restricted to mitochondria, and in all cases, colocalized with Mitotracker. Not all Mitotracker-stained mitochondria reacted with the antibodies, indicating that permeabilization, and thus antibody accessibility, was not complete. In our hands, antibodies raised against the recombinant protein (in this case anti-COX2B antisera) gave
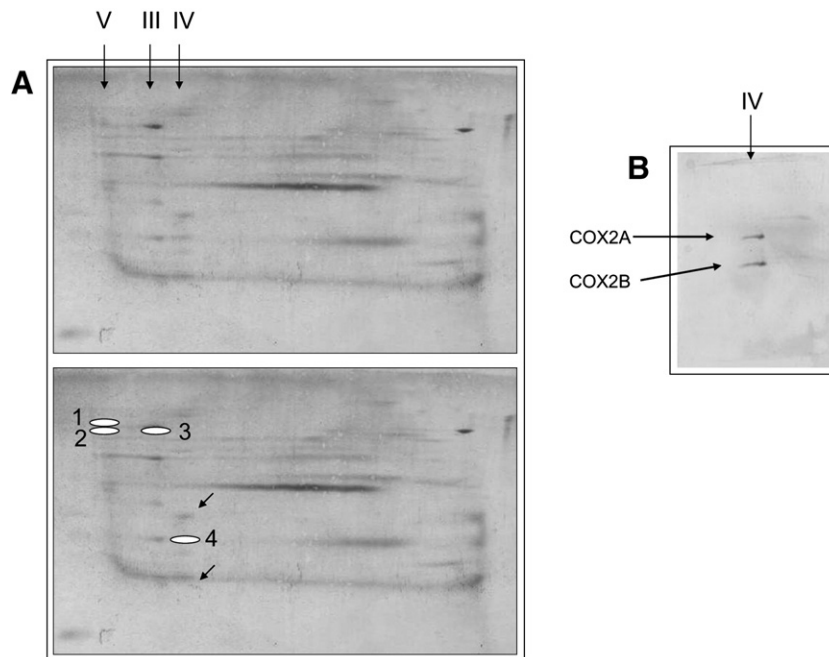

Fig. 6. Two-dimensional resolution of the mitochondria-enriched fraction from *T. gondii*. A) The identified OXPHOS complexes are indicated as V (F$_1$Fo-ATP synthase), III (*bc*$_1$ complex) and IV (cytochrome *c* oxidase). Mitochondria-enriched fractions were resolved on BN-PAGE followed by 2D-Tricine-SDS-PAGE. Bottom panel shows the spots that were identified as OXPHOS polypeptides by LC–MS/MS analysis. Spot 1, subunit α of F$_1$Fo-ATP synthase (complex V); spot 2, F$_1$Fo-ATP synthase subunit β; spot 3, subunit core 1 of *bc*$_1$ complex (complex III); and spot 4, subunit Vb of cytochrome *c* oxidase (complex IV). Arrows indicate the region where COX2A and COX2B were identified by immunoblotting. B) Immunoblot analysis of a 2D gel in the region where complex IV is located. The two immunoreactive bands with apparent molecular masses of 23.8 kDa (COX2A) and 13.2 kDa (COX2B), previously identified in whole call extracts and in the mitochondria-enriched fraction, migrated in association with complex IV.

much stronger signals and labeled more cells, than those raised against the antigenic peptide (anti-COX2A antisera).

To explore if the two immunoreactive bands (COX2A and COX2B) were actually associated with complex IV (cytochrome *c* oxidase), the *T. gondii* fraction enriched in mitochondria, obtained from Percoll gradients, was detergent-solubilized and analyzed by BN-PAGE followed by denaturing 2D Tricine-SDS-PAGE [21]. The polypeptide pattern of the obtained 2D gel is complex, exhibiting bands associated with high molecular weight OXPHOS complexes and abundant low-molecular mass proteins, some of which smear along the gel (Fig. 6). LC–MS/MS analysis of selected protein spots excised from the 2D gels revealed the presence of several secretion and surface components of *T. gondii* heavily contaminating the mitochondrial preparation (results not shown). Nevertheless, several other spots were unambiguously identified by LC–MS/MS analysis as mitochondrial polypeptides. Some of the identified proteins that pertained to OXPHOS complexes were the α and β subunits of F_1Fo-ATP synthase (GenBank 728921 and ABB17195 respectively), subunit I and the Rieske iron–sulfur protein of the bc_1 complex (GenBank AAF07940 and ToxoDB4.1_641.m00178), cytochrome *c* oxidase subunit Vb (GenBank CAJ20625), and the mitochondrial dihydrolipoamide dehydrogenase precursor (GenBank ABE76503). COX2A and COX2B polypeptides could not be identified in the spots analyzed by LC–MS/MS. Nevertheless, the abovementioned analysis allowed the identification of OXPHOS complexes, III, IV and V (Fig. 6). A blot of a similar 2D gel was probed with the anti-COX2A and anti-COX2B antibodies. Again, the two immunoreactive bands with apparent molecular masses of 23.8 kDa (COX2A) and 13.2 kDa (COX2B) were detected; both clearly migrated in association with mitochondrial complex IV.

4. Discussion

4.1. T. gondii cytochrome c oxidase contains a heterodimeric COX2 subunit

A previous biochemical study carried out with digitonin-permeabilized tachyzoites provided unequivocal evidence that respiration and OXPHOS are functional in *T. gondii*, and that a cyanide-sensitive cytochrome *c* oxidase is present in the parasite's mitochondria [28]. We thought of the importance to demonstrate that the presence of fragmented *Cox2a* and *Cox2b* genes in this apicomplexan parasite is not mere evolutionary remnants or pseudogenes, but fully functional genes whose corresponding products are imported into mitochondria, targeted to the inner mitochondrial membrane and assembled, giving rise to an active cytochrome *c* oxidase complex. In this work we demonstrated the presence of mature COX2A and COX2B subunits in *T. gondii* mitochondria by means of specific antibodies. Moreover, these polypeptides co-migrate with complex IV in BN-PAGE. The identification of COX2A (23.8 kDa) and COX2B (13.2 kDa) as distinct polypeptides indicates that subunit COX2 is a heterodimer in *T. gondii* like previously shown for the fragmented COX2 subunit of the chlorophycean algae *Polytomella* sp. [5] and *C. reinhardtii* [12].

BN-PAGE was the method of choice to address the study of mitochondrial components in an apicomplexan parasite. At this stage, secretory and surface proteins are still contaminating the parasite mitochondrial preparation, and further purification of mitochondria is required. Nevertheless, all the major OXPHOS complexes could be identified. The combination of BN-PAGE with mass spectrometry may allow the complete characterization of the *T. gondii* mitochondrial proteome in a near future.

4.2. Mitochondrial targeting sequences

Very few studies have addressed the targeting of *T. gondii* proteins to mitochondria and the structure of mitochondrial targeting sequences (MTS). Toursel et al. [29], demonstrated that the mitochondrial chaperone HSP60 contains an orthodox MTS. In contrast, Brydges and Carruthers [30] characterized an atypical mitochondrial superoxide dismutase with an unusual presequence, consisting of a hydrophobic segment similar to a signal peptide followed by a MTS. Parasite mitochondrial-targeted precursor proteins may exhibit unique MTS sequences that ensure delivery to the mitochondria and exclude targeting to other subcellular compartments (i.e., the apicoplast). The N-termini of neither COX2A nor COX2B could be determined in this work, therefore, no precise information on the processing sites of the corresponding precursor proteins could be obtained. Nevertheless, a striking difference between the apparent molecular mass of the *T. gondii* COX2A subunit (23.8 kDa) and its *Polytomella* sp. counterpart (13.0 kDa) was evident from the SDS-PAGE polypeptide patterns obtained using the same buffer system and the same set of molecular mass markers. It is known that highly hydrophobic polypeptides may exhibit anomalous mobility (usually reflecting lower apparent molecular masses) because of increased SDS binding to transmembrane regions [31]. Nevertheless, anomalous electrophoretic mobility could not account for such a large difference in apparent molecular masses (more than 10 kDa). An alternative explanation invokes a different processing site for the MTS in the parasite COX2A precursor protein. The N-terminus of the *Polytomella* sp. COX2A subunit has been determined experimentally, and therefore the exact processing site for the MTS is known [5]. The MTS of the COX2A protein from other organisms has been predicted on the basis of sequence similarity. If the *T. gondii* COX2A precursor protein would be processed at the same site as in its chlorophycean counterpart (as predicted only by sequence similarity alignment), a mature COX2A subunit of 14.8 kDa would be expected. To account for the experimentally observed *T. gondii* COX2A apparent molecular mass of 23.8 kDa, we hypothesize that the MTS of the COX2A precursor protein (accession number AF503914) is processed between residues H_{96} and Q_{97} (or somewhere in a region close to these residues), yielding a 23.8 kDa mature subunit. The MTS of the *T. gondii* COX2A precursor protein is longer than the algal one, and their amino acid sequences exhibit rather poor similarity. The processing site of the COX2A MTS, and thus the structure of the mature COX2A subunit in the parasite, may differ substantially from its counterpart in chlorophycean algae.

4.3. Evolutionary considerations

Nucleus-encoded *Cox2*a and *Cox2*b genes are present in a limited set of eukaryotic lineages: chlorophycean algae, apicomplexan parasites and dinoflagellates. Remarkably, apicomplexans and dinoflagellates form a well supported monophyletic group in eukaryotic phylogenies [32,33]. In phylogenetic reconstructions carried out with *Cox2a* and *Cox2b* gene sequences, chlorophycean algae tend to affiliate with apicomplexans and dinoflagellates, and appear far away from plant and other green algal sequences [7,15,10,34,35]. These results have been interpreted based on two different evolutionary scenarios. On one hand, it was suggested that apicomplexans acquired *Cox2a* and *Cox2b* genes from an endosymbiotic chlorophycean alga through horizontal gene transfer [7,15,36]. On the other hand, it has been argued that mitochondrial *cox2* genes fragmented and migrated to the nucleus in both the chlorophycean and the ancestor of dinoflagellates and apicomplexans in independent and unrelated events [34,35]. A recent phylogenomic analysis of the dinoflagellate *Karenia brevis* [37] revealed the existence of six plastid proteins derived from green algae. A reasonable explanation is that those dinoflagellate *green* genes derived from an ancestral green algal endosymbiont before the acquisition of the typical red-algal plastid of 'chromalveolates' [36,38]. Clearly, the evolutionary origin of fragmented, nucleus-encoded *Cox2* genes in apicomplexan parasites remains debatable. Nevertheless, the present work shows the presence of an active, heterodimeric COX2 subunit in an organism outside of the plant kingdom, and ascertains that the corresponding *Cox2a* and *Cox2b* genes are not evolutionary relics, but encode proteins of functional relevance to the parasites.

Acknowledgments

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