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Trypanosoma brucei:

Differential requirement of membrane potential for import of proteins into mitochondria in two developmental stages

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Abstract

Trypanosome alternative oxidase (TAO) and the cytochrome oxidase (COX) are two developmentally regulated terminal oxidases of the mitochondrial electron transport chain in *Trypanosoma brucei*. Here, we have compared the import of TAO and cytochrome oxidase subunit IV (COIV), two stage specific nuclear encoded mitochondrial proteins, into the bloodstream and procyclic form mitochondria of *T. brucei* to understand the import processes in two different developmental stages. Under *in vitro* conditions TAO and COIV were imported and processed into isolated mitochondria from both the bloodstream and procyclic forms. With mitochondria isolated from the procyclic form, the import of TAO and COIV was dependent on the mitochondrial inner membrane potential ($\Delta\psi$) and required protein(s) on the outer membrane. Import of these proteins also depended on the presence of both internal and external ATP. However, import of TAO and COIV into isolated mitochondria from the bloodstream form was not inhibited after the mitochondrial $\Delta\psi$ was dissipated by valinomycin, CCCP, or valinomycin and oligomycin in combination. In contrast, import of these proteins into bloodstream mitochondria was abolished after the hydrolysis of ATP by apyrase or removal of the ATP and ATP-generating system, suggesting that import is dependent on the presence of external ATP. Together, these data suggest that nuclear encoded proteins such as TAO and COIV are imported in the mitochondria of the bloodstream and the procyclic forms via different mechanism. Differential import conditions of nuclear encoded mitochondrial proteins of *T. brucei* possibly help it to adapt to different life forms.

Keywords

Trypanosoma brucei; Mitochondrial protein import; Membrane potential; Trypanosome alternative oxidase

1. Introduction

Trypanosoma brucei is a group of unicellular parasitic protozoa that cause a fatal disease in human beings and domestic animals in Sub-Saharan Africa known as African trypanosomiasis (WHO report; Vickerman, 1986). During its transmission from one mammal to another by the insect vector, the tsetse fly, *T. brucei* changes its morphology, ultrastructural organization and various biochemical characteristics, particularly its mitochondrial activities (Schneider, 2001; Lukes et al., 2005). In the mammalian host, *T. brucei* stays in the blood stream and

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utilizes glucose as its sole source of energy. Thus, the bloodstream form of *T. brucei* reduces much of its mitochondrial function. This form does not have any cytochromes and respire via a non-cytochrome mediated terminal oxidase, known as trypanosome alternative oxidase (TAO) (Chaudhuri et al., 2006). TAO-mediated mitochondrial electron transport does not generate $\Delta\psi$ across mitochondrial inner membrane. Thus, the bloodstream form does not produce ATP by oxidative phosphorylation. Instead, it hydrolyzes ATP to generate a mitochondrial $\Delta\psi$ via ATPase (Nolan and Voorheis, 1992; Vercesi et al., 1992; Schnauffer et al., 2005; Brown et al., 2006). It has been shown that the bloodstream form possesses a lower mitochondrial $\Delta\psi$ and a lower level of ATP in the matrix compared to the procyclic form (Williams, 1994; Nolan and Voorheis, 2000).

Following a blood meal by the insect, the bloodstream form differentiates to the procyclic form in the fly's alimentary system. Here, it develops its mitochondrial activities and utilizes primarily amino acids as its energy source (Bouchud-Alleman and Schneider, 2002; Besteiro et al., 2005). The procyclic form reduces TAO expression about 100 fold by decreasing the stability of the transcript (Chaudhuri et al., 2002). On the other hand it develops a full complement of the standard cytochrome-dependent electron transport pathway (Chaudhuri et al., 2006). Electron transport through this pathway is coupled with proton translocation across the mitochondrial inner membrane. ATP is produced in the mitochondria both by substrate level and oxidative phosphorylation (Bouchud-Alleman and Schneider, 2002; Besteiro et al., 2005).

The cytochrome-dependent respiratory complexes consist of multiple subunits. Most of the components of these complexes are nuclear encoded and must be imported to the mitochondria after their synthesis (Zee and Glerum, 2006). TAO is also a nuclear encoded mitochondrial protein and is the only protein so far known that is over expressed in the rudimentary form of the mitochondrion found in the bloodstream trypanosomes (Chaudhuri et al., 2002). The import mechanism in the mitochondria has been studied for a few components of the cytochrome bc1 complex and NADH dehydrogenase subunit k (Ndh K) in *T. brucei* (Priest and Hajduk, 1996; Bertrand and Hajduk, 2000; Priest and Hajduk, 2003). However, mitochondrial import of TAO has not been explored.

Inner membrane potential is the major driving force for import of most mitochondrial matrix or inner membrane proteins (Esaki et al., 1999; Pfanner and Geissler, 2001; Priest and Hajduk, 2003; Endo et al., 2003), except for a very few proteins, import of which does not depend on $\Delta\psi$, such as yeast mitochondrial transcription factor (Mtf1P) (Biswas and Getz 2002). In addition, for complete translocation of proteins across both membranes ATP must be hydrolyzed in the matrix to provide the energy for physical movement of the precursor proteins through the import channel (Horst et al., 1996; Strub et al., 2000). In contrast to inner membrane and matrix localized proteins, mitochondrial outer membrane proteins and many proteins in the inter-membrane space require neither $\Delta\psi$ nor ATP hydrolysis in the matrix for their import (Pfanner and Geissler, 2001; Endo et al., 2003; Neupert and Herrmann, 2007). Import of proteins to all four sub-compartments of mitochondria, such as the outer and inner membrane, intermembrane space, and matrix, requires receptors on the mitochondrial outer surface and specific translocator complexes in both membranes (Pfanner and Geissler, 2001; Endo et al., 2003; Neupert and Herrmann, 2007). To cross the mitochondrial membranes, proteins must be in an import-competent state, which is achieved by cooperation with cytosolic chaperone proteins in presence of ATP (Pfanner and Geissler, 2001; Endo et al., 2003; Young et al., 2003; Asai et al., 2004). All these parameters of mitochondrial protein import can be manipulated under *in vitro* conditions during import of radiolabelled precursor proteins into isolated mitochondria (Priest and Hajduk, 2003; Sherman et al., 2005; Wiedemann et al., 2006). Thus, a cell-free mitochondrial protein import system serves as an ideal tool to compare the import of different proteins into mitochondria.

Here, we have compared different criteria for import of the TAO and COIV precursor proteins into mitochondria of the procyclic and the bloodstream forms of *T. brucei in vitro*. Our results suggest that these stage-specific proteins are imported into mitochondria from both forms. However, the requirement of $\Delta\psi$ for import of TAO and COIV into the mitochondria of the bloodstream and the procyclic forms are different.

2. Materials and Methods

2.1. Cells

The procyclic form of *Trypanosoma brucei* 427 was grown in SDM-79 medium containing 10% fetal bovine serum and the bloodstream form cells were maintained in HMI-9 media supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 10% serum plus (JRH Biosciences) (Hirumi and Hirumi, 1984). For isolation of mitochondria, the bloodstream form cells were grown *in vivo* using Sprague-Dawley rats. The parasites were separated from the blood collected from infected rats by diethyl amino-ethyl (DEAE) cellulose chromatography as described (Chaudhuri et al., 1995).

2.2. Mitochondria isolation

Mitochondria were isolated from the parasite after lysis by nitrogen cavitation in isotonic buffer, as described earlier (Chaudhuri and Nargang, 2003). Briefly, the cells were washed with a buffer containing 0.15 M NaCl, 20 mM glucose, and 20 mM NaH₂PO₄, pH 7.4 and resuspended at a density of 1×10^9 cells/ml in SME buffer (0.25 M sucrose, 20 mM MOPS/KOH, pH 7.2, 2 mM EDTA, 1 mM PMSF, 0.5 μ g/ml leupeptin and 1 μ g/ml pepstatin). The cells were equilibrated at 700 psi N₂ for 15 min in a nitrogen cavitation bomb (minibomb cell disruption chamber; Kontes, Vineland, NJ). Subsequent release from the bomb resulted in the disruption of more than 90% of cells. The lysate was diluted with one volume of the same buffer and the mitochondrial fraction was isolated by differential centrifugation, as described previously (Priest and Hajduk, 1994; Chaudhuri and Nargang, 2003). Cell lysate was centrifuged first at 1,500 g for 10 min to remove cell debris, unbroken cells, and nucleus. The supernatant were then centrifuged at 15,000 g for 10 min to obtain a crude mitochondrial pellet. The process was repeated once to obtain a homogeneous suspension of mitochondrial fraction. For isolation of mitochondria from the bloodstream form of *T. brucei*, cells were grown *in vivo* using rat as the model system. Parasites were isolated from infected rat blood, lysed by N₂-cavitation and mitochondria were purified as described above. For further purification, mitochondria were resuspended in 50% percol and centrifuged at 100,000 g for 60 minute using a linear gradient of 20 to 35% percol (Sabatini et al., 1998). After centrifugation, mitochondrial vesicles floated at the middle of the gradient were collected and washed thoroughly with SME buffer. The isolated mitochondria were stored at a protein concentration of 5 mg/ml in MOPS/KOH buffer, pH 7.2, containing 50% glycerol at -70 °C. Before use, mitochondria were washed twice with 9 volumes of SME buffer to remove glycerol.

2.3. In vitro transcription and translation

The TAO open reading frame (ORF) was PCR amplified using primers; TAO^{For} : 5' AGAAGCTTATGTTTCGTAAC3' and TAO^{Rev}: 5'AAGAATTCTTACTCGTGTTTG3' containing appropriate restriction sites (underlined) at the 5' ends. TAO cDNA (pTAO25) (Chaudhuri et al., 1998) was used as a template. Similarly, the ORF for COIV (Tb927.1.4100) (Gene DB: www.genedb.org/genedb/trypan) was amplified from *T. brucei* genomic DNA using primers; COIV^{For}: 5'AGAAGCTTATGTTTGCTCGCCGCT3' and COIV^{Rev}: 5' AAGAATTCCCTAAATCTTGTGTTGA3'. The PCR product was subcloned in pGEM 4 vector at the site of HindIII/EcoRI. Radiolabeled precursor protein was synthesized *in vitro* using a coupled transcription/translation rabbit reticulocyte system (TNT, Promega) according to the manufacturer's protocol using ³⁵S methionine (DuPont NEN) as the label.

2.4. Import assay

Labeled precursor proteins were used for *in vitro* import into isolated mitochondria from *T. brucei* as described (Priest and Hajduk, 1996; Bertrand and Hajduk, 2000; Priest and Hajduk, 2003) with a few modifications. Mitochondria (100 µg) were washed with 9 volumes of SME buffer and resuspended in 90 µl of import buffer (0.25 M sucrose, 80 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 1.0 mg/ml fatty acid free bovine serum albumin and 10 mM MOPS/KOH at pH 7.2). The mitochondrial suspension was mixed with 10 µl of rabbit reticulocyte translation mixture containing a radiolabeled precursor protein and incubated at room temperature for up to 30 min. Additional ATP was provided at a final concentration of 2 mM and 10 mM creatine phosphate and 0.1 mg/ml creatine phosphokinase were added as an ATP regeneration system. 8 mM potassium ascorbate, 0.2 mM N,N,N',N'-tetramethylphenylenediamine, and 5 mM NADH were added to energize the mitochondria and provide reducing equivalents. Following import, the mitochondria were treated with proteinase K (50 µg/ml) at 0 °C for 15 min to remove the labeled protein that is bound to the membrane but not imported. PMSF (2.0 mM) was then added to inhibit proteinase K and the mitochondrial fraction was re-isolated by centrifugation. Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membrane as described (Towbin et al., 1979). After transfer, the blot was dried at 37 °C for 30 min and exposed to X-ray films (Biomax Film, Kodak) for detection of radioactive proteins. Imported protein bands were quantitated by densitometric scanning (Bio-RAD Model GS-700) using Quantity One software program. For some experiments, the same blot was immunodecorated with mitochondrial Hsp70 (mHsp70) antibody (Effron et al., 1993) and appropriate secondary antibody conjugated with horseradish peroxidase. Immunoblots were developed with the ECL detection system according to the manufacturer's protocol (GE healthcare).

2.5. Additional treatment of mitochondria before and after import

For some experiments the post-import mitochondrial fraction was treated with Na₂CO₃ (0.1 M) for 30 min at 4 °C and was centrifuged at 12,000 g for 10 min to separate integral membrane and soluble proteins (Chaudhuri and Nargang, 2003). To test for the requirement of surface receptors in import, proteins from the outer surface were removed by treating mitochondria with trypsin (100 µg/ml) for 15 min on ice and stopping the digestion with soybean trypsin inhibitor (STI) added at a final concentration of 1.2 mg/ml. In a few cases, mitochondria were also treated with Triton X-100 (1%) in combination with proteinase K (50 µg/ml) following import to show that the mature protein was not protease resistant when mitochondrial membranes were dissolved by detergent treatment, which provides access to proteinase K. Pre-treatment of mitochondria with valinomycin, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), oligomycin, and atractyloside (purchased from Sigma) was performed by adding these reagents to the import reaction from 100 x stock solutions in DMSO to a final concentration of 0-15, 0-150, 30, and 400 µM respectively, for 5 min at room temperature before radiolabeled precursor proteins were added. The rabbit-reticulocyte lysate were treated with apyrase (Sigma) at a concentration of 10 units/ml for 30 min at 25 °C to hydrolyze ATP.

2.6. Mitotracker staining and confocal microscopy

Mitochondria isolated from the procyclic and the bloodstream forms were incubated on ice at 1 mg/ml protein concentration in the presence and absence of valinomycin (0.5 µM) plus CCCP (50 µM) for 15 min. Mitotracker™ green FM (Molecular probe) was dissolved in dimethyl sulfoxide at a concentration of 1 mM and added to mitochondria at a final concentration of 5 µM. The mixture was incubated for 20 min at 27 °C and 37 °C for the procyclic and the bloodstream form mitochondria, respectively. After that mitochondria were reisolated by centrifugation, washed and incubated in SEM for additional 30 min. Mitochondria were then washed twice with SEM and fixed in 4% paraformaldehyde at 4 °C for 15 min. Mitochondrial

suspension was spread on poly-lysine coated slides. The slides were mounted in Fluoromount G and images were acquired with a Nikon TE2000-U C1 laser scanning confocal microscope outfitted with a 60×1.4 N.A. Plan Apo oil immersion objective lens. MitoTracker green was excited with a 488 nm argon laser, and the emitted light was collected using a 505-550 nm bandpass filter. All images were collected with exactly the same detector gain, laser strength, and number of scans in order for the data to be examined for differences in fluorescence intensity.

2.7. Measurement of membrane potential

The membrane potential of isolated mitochondria was measured using potential-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide (DiSC3) as described (Bertrand and Hajduk, 2000). Briefly, a 2 mM stock of DiSC3 in ethanol was diluted in SEMP to a final concentration of 4 μ M. Fluorescence was determined using a spectrophotometer (Horiba Jobin Yvon Fluoromax-3) at wavelength 620 nm for excitation and 670 nm for emission in the presence and absence of mitochondrial suspension (1 mg/ml). The membrane potential was dissipated by the addition of valinomycin at a final concentration of 0.5 μ M. The difference in the fluorescence intensity after addition of mitochondria represents a rough assessment of the membrane potential ($\Delta\psi$).

2.8. Electron microscopy

Mitochondria were fixed in 2% (vol/vol) glutaraldehyde, 2% (wt/vol) paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.2. The mitochondria were then washed in SCB, postfixed with 1% (wt/vol) osmium tetroxide in SCB, stained with 0.5% aqueous magnesium uranyl acetate, dehydrated, and embedded in Spurr's resin (Hyde et al., 2003). Blocks were sectioned at 50 to 70 nm thickness and stained in 5% (wt/vol) uranyl acetate in 1% acetic acid, 0.4% lead citrate in 0.1 N NaOH. Section grids were inserted into the FEI CM12 twin lense 420 transmission electron Microscope (FEI) to capture images.

2.9. Measurement of ATP concentration

ATP concentration was measured using the ATP Bioluminescence assay kit (Invitrogen) as described (Allemann and Schneider, 2000). Mitochondria were suspended in import buffer at the concentration of 1 mg/ml and incubated at room temperature for 15 min in the presence and absence of oligomycin (30 μ M). Following the incubation the samples (100 μ l) were treated with 2.5 μ l of 60% perchloric acid and immediately vortexed. Samples were incubated on ice for 30 min and centrifuged at 13,000 rpm for 5 min. Supernatants were collected in fresh tubes and 20 μ l of 1N KOH was added to each tube to neutralize the acid. Samples were centrifuged again and the resultant supernatants were used for estimation of ATP concentration by luciferase ATP assay reagents according to the manufacturer's protocol. Briefly, 10 μ l of sample were mixed with 90 μ l of standard reaction solution containing 25 mM Tricine buffer, pH 7.8, 5 mM MgSO₄, 0.1 mM EDTA, 1 mM DTT, 0.5 mM D-Luciferin, and 1.5 μ g/ml of firefly luciferase. ATP standards in the range of 0-100 nM were used to generate a standard curve. The fluorescent intensity was measured with a luminometer using a 1 second delay and an integration time of 1 to 10 sec. The blank (no ATP) was subtracted from each reading and ATP concentrations for each sample were presented as the percent of untreated control.

3. Results

3.1. TAO is imported into isolated mitochondria from the procyclic and the bloodstream forms

Protein sorting signals and localization sites (PSORT) and prediction of mitochondrial protein (Mitoprot) analysis of the TAO open reading frame (ORF) predicted that TAO possesses a

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cleavable N-terminal mitochondrial targeting signal of 24 amino acids (Gene DB: www.genedb.org/genedb/trypan). Furthermore, the relative size of TAO expressed in *T. brucei* mitochondria was 2-3 kDa smaller than the molecular mass calculated from its cDNA sequence (Chaudhuri et al., 1998), which suggests that TAO possesses a cleavable presequence. To understand the import process of TAO into mitochondria we have standardized an *in vitro* mitochondrial protein import system in our laboratory based on previously published reports (Priest and Hajduk, 1996; Bertrand and Hajduk, 2000; Priest and Hajduk, 2003; Shermann et al., 2005; Wiedemann et al., 2006). Using this *in vitro* import technique we found that TAO was imported into procyclic mitochondria and processed to its mature form. The 35 kDa precursor TAO protein, the major product in the TNT lysate, specifically bound to mitochondria and was processed to a 33 kDa matured protein. Post-import proteinase K treatment removed the precursor protein but the processed protein was protected from protease digestion, suggesting that it is the mature form of TAO that is fully translocated to the interior of mitochondria (Fig. 1A). Several other radiolabeled proteins of different sizes were found in our *in vitro* translation reaction products (Input lane in Fig 1 A). These are lesser intense than the major 35 kDa protein and possibly generated either due to use of internal methionine as the start site or due to degradation of the major product. Our import results clearly showed that these lower molecular mass proteins were neither imported into mitochondria nor protected from the post-import proteinase K digestion, indicating the specificity of this import process. Pretreatment of mitochondria with valinomycin disrupted mitochondrial $\Delta\psi$ and inhibited formation of the mature product, however, binding of the precursor protein to mitochondria was not inhibited (Fig. 1B). This indicates that when $\Delta\psi$ is dissipated TAO can no longer enter into mitochondria. Thus, the import of TAO into mitochondria from the procyclic form is dependent on $\Delta\psi$.

We next conducted a series of experiments to further characterize the import of TAO into mitochondria. We pretreated mitochondria with trypsin to remove any externally exposed outer membrane proteins. The trypsin treatment abolished TAO binding and import, suggesting that TAO depends on mitochondrial outer membrane receptors/translocators for its entry into mitochondria (Fig. 1C). In addition, when the mitochondria were rendered permeable by Triton X-100 after the import assay and treated with proteinase K, the mature TAO protein was digested, indicating that during import the processed product is protected inside mitochondria (Fig. 1C). For each experiment, after autoradiography, the same blot was immunodecorated with antibodies against mHsp70 to confirm that protease digestion did not disrupt mitochondria. However, when the membrane was solubilized by the addition of detergent, TAO and Hsp70 were both susceptible to proteinase K digestion. To understand if TAO is integrated into the mitochondrial membrane after being imported, we extracted mitochondria with Na_2CO_3 and analyzed the supernatant and pellet fractions for the presence of radiolabeled TAO. TAO was found exclusively in the membrane pellet (Fig. 1D). In contrast, the mitochondrial matrix protein mHsp70 was exclusively present in the supernatant, as expected. This suggests that TAO is properly integrated into the mitochondrial membrane after *in vitro* import. All together, our data reveal that TAO possesses a cleavable presequence and that TAO was imported into isolated procyclic mitochondria using our *in vitro* assay system.

We also tested the import of TAO into mitochondria isolated from the bloodstream form using the *in vitro* assay. Results showed that TAO was imported into the bloodstream mitochondria and was processed to its matured form under *in vitro* conditions. The import was poor at 25 °C but increased about 2-3 fold as the incubation temperature was increased to 30 °C and 37 °C (Fig. 2A & B). Since the normal growth temperature of the bloodstream form is 37 °C, we performed all further *in vitro* import assays with bloodstream form mitochondria at this temperature. When the mitochondria were allowed to import precursor proteins and then treated with proteinase K, most of the precursor proteins were digested; however, the processed product remained intact, suggesting that some of the precursor proteins had fully entered the

mitochondria and been processed to their mature form (Fig. 2C). However, unlike with the mitochondria from the procyclic form, the precursor protein was not completely digested by post-import protease treatment, which could possibly be due to incomplete processing of the precursor protein inside mitochondria under this condition. As with the mitochondria from procyclic trypanosomes, the mature protein was degraded when the mitochondria were made permeable with Triton X-100 (Fig. 2C). The imported protein was resistant to extraction by Na_2CO_3 (Fig. 2D), suggesting it is membrane-integrated, as was true in the procyclic mitochondria (Fig. 1D).

3.2. TAO and COIV are imported into bloodstream form mitochondria in the presence of membrane potential inhibitors

It has been reported previously that mitochondria isolated from *T. brucei* by isotonic lysis of the cells followed by differential centrifugation maintain mitochondrial $\Delta\psi$ and intact double membranes (Hauser et al., 1996; Bertrand and Hajduk, 2000). To verify that the mitochondrial preparation in our hands also maintains these qualities, we assessed the membrane potential by probing mitochondria with a fluorescent dye MitoTracker™ green. MitoTracker™ green is taken up by mitochondria in a $\Delta\psi$ dependent manner (Vassella et al., 1997). Valinomycin is a potassium ionophore, and CCCP is a protonophore, both of which dissipate mitochondrial membrane potential (Gallo et al., 1984). The bloodstream and the procyclic form mitochondria were stained with mitotracker showing that both forms possess mitochondrial $\Delta\psi$ and treatment of mitochondria with valinomycin (0.5 μM) and CCCP (50 μM) disrupt $\Delta\psi$ (Fig. 3A). A similar results were observed when mitochondria were treated individually either with valinomycin or CCCP.

We also assess the $\Delta\psi$ by measuring uptake of a fluorescent probe DisC3 by isolated mitochondria. DisC3 is taken up by mitochondria in a membrane potential dependent manner (Geissler et al., 2001). The fluorescence intensity of DisC3 fell sharply when we added the bloodstream and the procyclic form mitochondria indicating that the isolated mitochondria possess a $\Delta\psi$ (Fig. 3B). Addition of both the procyclic and bloodstream mitochondria showed a drop in the fluorescence intensity. The drop was reversed in both cases after addition of valinomycin. This suggests that valinomycin disrupts mitochondrial $\Delta\psi$ and the dye was released. In addition, we also checked our mitochondrial preparation by electron microscopy to verify that the mitochondrial vesicles possess a double membrane. Results indicate that mitochondria isolated from both the procyclic and bloodstream forms possess double membranes. The procyclic mitochondria show a more cristate structure than the bloodstream forms as expected (Fig. 3C).

To verify the requirement of mitochondrial membrane potential during import of TAO, we pretreated mitochondria with various concentrations of valinomycin and CCCP. Results showed that the precursor protein import is completely inhibited in the procyclic form mitochondria. However, import of TAO into the bloodstream form mitochondria was not inhibited in the presence of either valinomycin or CCCP. Pre-treatment of procyclic mitochondria with 2.5 μM valinomycin or 25 μM CCCP is sufficient to block the import of TAO. However, import in the bloodstream form was not inhibited even at 15 μM valinomycin or 150 μM CCCP (Fig. 4A). A lower molecular size band was found in all samples, which is a non-specific product generated during *in vitro* translation. This non-specific product did not enter into mitochondria as we showed in Fig. 1A.

For further investigation, we selected another developmentally regulated nuclear encoded mitochondrial protein, COIV of *T. brucei*. TAO is up regulated in the bloodstream form, whereas, COIV protein is not found in the bloodstream form but is abundant in the procyclic form. *T. brucei* COIV has 44 amino acids predicted cleavable presequence (Gene DB). We have also found that after import the precursor protein is cleaved and produce the matured form

of the protein. Interestingly, COIV import also follows the similar pattern as of TAO. Import of COIV precursor protein is inhibited by valinomycin and CCCP in the procyclic form mitochondria. However, neither CCCP nor valinomycin has any effect on COIV import into the bloodstream mitochondria (Fig. 4B).

Next we wanted to verify if the imported proteins entered into bloodstream form mitochondria and not in other vesicles. After *in vitro* import of radiolabeled precursor TAO we purified mitochondria by percol floatation gradient centrifugation (Fig. 5). The top, bottom, and the middle fraction (1.0 cm each) of the gradient were analyzed for the presence of radiolabeled TAO protein. We found that the bound precursor and the matured TAO proteins were primarily accumulated in the middle fraction, where mitochondrial vesicles are expected to be present. The blot was immunodecorated sequentially with TAO and tubulin antibodies. Results showed that the endogenous TAO is exclusively present in the same fraction, where imported radiolabeled TAO was found. Whereas, tubulin is present almost at similar levels in the top and middle layers of the gradient and also present at a lower level in the bottom fraction. Tubulin is not a mitochondrial protein but it is always present as a membrane associated protein in the mitochondrial fraction. These results clearly showed that TAO is imported into the bloodstream form mitochondria in a membrane potential independent manner.

3.3. Import kinetics of TAO and COIV in the procyclic and the bloodstream form mitochondria

Next we compared the import of TAO and COIV in isolated mitochondria from the bloodstream and procyclic forms. Assay results showed that both TAO and COIV are imported into mitochondria of the procyclic and the bloodstream forms *in vitro*, in a time-dependent manner (Fig. 6). In the procyclic form the mature TAO protein accumulated maximum within 20 min (Fig. 6A & C). In contrast, in the bloodstream form maximum import was reached earlier at 10 min. The amount of imported protein was significantly less in the bloodstream mitochondria in comparison to the procyclic mitochondria. Binding of the precursor TAO to isolated procyclic and the bloodstream mitochondria also follows similar pattern to its matured product in respective mitochondria. Quantitative estimation of the precursor and the mature protein at different time points revealed that the $t_{1/2}$ for TAO import into mitochondria of the procyclic and the bloodstream forms was about 5 and 3.5 min, respectively. A similar kinetic pattern was observed when TAO was imported at 25 °C instead of 37 °C into mitochondria of the bloodstream form (not shown). Together, it showed that TAO was imported in a time dependent manner into mitochondria from both the procyclic and bloodstream forms. Import reaches maximum in the bloodstream mitochondria quickly than in procyclic mitochondria.

Similar to TAO, the COIV was also imported into mitochondria isolated from the procyclic and bloodstream forms. The mature protein accumulates in the mitochondria gradually with time during import. A maximum import reached at 20 min in both forms. The $t_{1/2}$ was also very similar (7.5 min) for COIV import in the procyclic and bloodstream mitochondria (Fig. 6B & D).

3.4. TAO and COIV import into the mitochondria of the procyclic and bloodstream forms depend on the presence of external and internal ATP

It is known in other systems that ATP is necessary for the nascent precursor protein to interact with chaperone proteins such as Hsp70 and Hsp90 to remain in a partially unfolded and import-competent state (Geissler et al., 2001; Young et al, 2003; Asai et al., 2004). We asked whether import of TAO and COIV in the bloodstream and procyclic forms mitochondria require external ATP. To test the requirement of external ATP we treated the reticulocyte lysate containing radiolabeled precursor protein with apyrase before adding to the import reaction to hydrolyze ATP. Under this condition the precursor protein import was mostly inhibited in both the procyclic and bloodstream mitochondria (Fig. 7A, B & D). These results indicate that TAO

and COIV must be in an import competent state for entry into mitochondria in both the procyclic and bloodstream forms.

Mitochondrial ADP/ATP carrier protein (AAC) in the procyclic form exports ATP from mitochondrial matrix. Thus, pretreatment of mitochondria with atractyloside, an inhibitor of AAC, blocks export of matrix ATP from mitochondria (Priest and Hajduk, 2003) and reduces external ATP. We treated the procyclic mitochondria with atractyloside and performed import assay. Results showed that treatment of mitochondria with atractyloside alone did not inhibit the import of TAO and COIV into procyclic mitochondria (Fig. 7A & D). This could be because there is sufficient ATP and ATP regeneration capacity in the import buffer to sustain precursor protein import. Once the ATP and the ATP regeneration system were removed from the import buffer, TAO import was completely inhibited into the procyclic form mitochondria (Fig. 7A & D). In the bloodstream form mitochondria COIV import was inhibited more than 50% (Fig. 7D) and TAO was totally inhibited (not shown) in the absence of ATP regeneration system. This indicates that the presence of external ATP is critical for TAO and COIV import.

In addition to the requirement of external ATP, hydrolysis of ATP in the matrix is also essential for driving import of matrix and inner membrane targeted proteins (Pfanner and Geissler, 2001; Endo et al., 2003; Neupert and Herrmann, 2007). To determine whether matrix ATP is required for import of TAO and COIV into mitochondria of the procyclic form we treated mitochondria with oligomycin, an inhibitor of mitochondrial ATP-synthase (Penefsky, 1979). The ATP concentration before and after oligomycin treatment was assessed by a luciferase based assay system as described in the section 2.8. In procyclic mitochondria ATP concentration was reduced about 20% after oligomycin treatment (Fig. 7C). This small reduction in ATP concentration is possibly because of partial inhibition of ATP-synthase activity by oligomycin and also because of the presence of substrate level phosphorylation, as has been reported previously (Coustou V et al., 2003). However, in procyclic mitochondria import of TAO and COIV proteins was completely inhibited after treatment with oligomycin suggesting that a partial reduction of ATP level is detrimental for import of proteins (Fig. 7A & D).

The scenario for mitochondrial ADP/ATP metabolism and generation of membrane potential is different in the bloodstream form than the procyclic form. In the bloodstream form, ATP is imported into matrix by AAC, where it is hydrolyzed by ATPase to generate membrane potential (Schnauffer et al., 2005). Thus, it is expected that inhibition of mitochondrial ATPase by oligomycin reduces inner membrane potential but increased the ATP level in the matrix. In our luciferase-based assay, we also found that the ATP level was increased about 1.6 fold after treatment of bloodstream mitochondria with oligomycin (Fig. 7C). It has been verified recently that a reduction of mitochondrial ATPase by RNAi significantly reduces mitochondrial membrane potential in the bloodstream form (Schnauffer et al., 2005; Brown et al., 2006). We have found that import of TAO and COIV is not inhibited when the bloodstream mitochondria are pretreated with oligomycin (Fig. 7B & D), indicating further that TAO and COIV import in the bloodstream form mitochondria does not depend on $\Delta\psi$. We also treated mitochondria with oligomycin in addition to valinomycin for complete disruption of $\Delta\psi$, but no inhibition on import was observed (Fig. 7B & C). These results strongly suggest that TAO and COIV import in the bloodstream form mitochondria is independent of $\Delta\psi$. We did not see any significant change in the total ATP concentration when the bloodstream form mitochondria were treated with atractyloside. This is also correlated with our import results that atractyloside had no effect on import of TAO and COIV into the bloodstream form mitochondria. Overall, it showed that precursor proteins TAO and COIV are imported by different mechanism in two developmental forms of *T. brucei*.

Discussion

We have demonstrated that two stage-specific nuclear encoded mitochondrial proteins from *T. brucei* are imported *in vitro* into mitochondria from both developmental forms. Furthermore, the same precursor proteins, which depend on the $\Delta\psi$ for import into the procyclic mitochondria, can be imported in the bloodstream form mitochondria in the absence of $\Delta\psi$, suggesting that an alternate mechanism of import is possibly present in the mitochondria of the later form. This flexibility in import conditions is possibly needed to adjust with the condition of mitochondria in different life forms in *T. brucei*.

Like alternative oxidases from plants and fungi, TAO is thought to be a peripheral inner mitochondrial membrane protein that possesses two hydrophobic stretches that are inserted into the inner leaflet of the lipid bilayer of mitochondrial inner membrane; thus, both the N- and C-terminals of the protein are placed in the matrix (Chaudhuri et al., 2006). On the other hand, COIV is not a transmembrane protein, but it is peripherally attached to cytochrome oxidase complex located on the mitochondrial inner membrane (Maslov et al., 2002). TAO and COIV each possess a cleavable N-terminal targeting signal. The predicted cleavage sites of the TAO and COIV presequences are after 24 and 44 amino acids at the N-terminal, respectively. These cleavage sites match with the R-2 motif; xRx↓x(S/x) that is recognized by mitochondrial processing peptidase (MPP) (Gakho et al., 2002). This prediction is correlated with our findings that the protease protected mature proteins, TAO and COIV generated in the import reactions are 2 and 4 kDa smaller than their precursor forms, respectively.

Import of TAO and COIV in both the bloodstream and procyclic mitochondria is strictly dependent on the presence of ATP in the rabbit reticulocyte lysate and also in the import buffer. Thus, ATP hydrolysis must occur in the cytosol to keep the nascent precursor protein in a partially unfolded state. The import of these proteins also occurs in a time-dependent manner into both the procyclic and the bloodstream form mitochondria. Under *in vitro* conditions, the amount of TAO imported into the bloodstream form mitochondria was relatively lower than that in the procyclic mitochondria. This is somewhat unexpected, because TAO is more abundant in the bloodstream than in the procyclic form. Thus, a lower import efficiency of the bloodstream form mitochondria for TAO is possibly because of the *in vitro* conditions. The cytosolic factor(s) essential for efficient import of TAO in the bloodstream form mitochondrion may have been deleted during preparation and account for the smaller amount of TAO import. The $t_{1/2}$ for TAO import is shorter in the bloodstream form in comparison to the procyclic form mitochondria. Similar kinetic pattern has also been reported for import of the Ndh K into isolated mitochondria from the bloodstream and procyclic forms (Bertrand and Hajduk, 2000). It is possible that the bloodstream mitochondria possess lesser number of preprotein receptor/translocator complexes in comparison to that in the procyclic mitochondria; thus, in the bloodstream form the receptors became saturated quickly with presequence during *in vitro* import.

Most interestingly, we have found that the import of TAO as well as COIV in the mitochondria of the bloodstream form do not depend on $\Delta\psi$. It has been reported previously that Ndh K, a constitutively expressed nuclear encoded protein is imported into mitochondria of the bloodstream form in a $\Delta\psi$ -dependent manner (Bertrand and Hajduk, 2000). Thus, the phenomenon we observed may be protein specific, which possibly depends on the final destination of the protein and the type of targeting signals they have. In higher eukaryotes, inner mitochondrial membrane proteins containing N-terminal targeting signal are generally imported by two different mechanisms: 1) the conservative sorting and 2) the stop-transfer (Pfanner and Geissler, 2001; Endo et al., 2003). In the conservative sorting pathway, the protein is first targeted to mitochondrial matrix by its N-terminal targeting signal, this signal is cleaved by MPP and another signal consisting of primarily hydrophobic amino acid residues flanked

by charged residues and located just downstream of the N-terminal targeting signal, re-directs the protein to mitochondrial inner membrane. The second signal is then also cleaved by mitochondrial intermediate peptidase (MIP) or inner membrane peptidase (IMP) (Gakho et al., 2002). A similar bipartite signal and the conservative sorting mechanism have been found for Rieske iron-sulfur protein (ISP) in *T. brucei* (Priest and Hajduk, 1996). TAO does not have a bipartite signal and also we have seen that the TAO presequence is cleaved only once in both the bloodstream and the procyclic form. Thus, TAO is possibly not imported by the conservative sorting mechanism.

On the other hand, in stop-transfer mechanism, the precursor protein possesses a hydrophobic membrane binding region located downstream of the N-terminal targeting signal. The protein is targeted via its N-terminal signal and when the membrane spanning region reaches the translocase of mitochondrial inner membrane (TIM) complex, it laterally opens and releases the protein in the membrane (Pfanner and Geissler, 2001; Endo et al., 2003; Neupert and Herrmann, 2007). This process requires both membrane potential and ATP hydrolysis inside matrix. It can be speculated that TAO follows this pathway, particularly in the procyclic mitochondria. After entry, the presequence is cleaved by MPP and proteins refold and assemble on the inner membrane. The precursor COIV also processed once during import. It depends on $\Delta\psi$ and matrix ATP for its import. This protein is likely translocated via the matrix targeted protein import pathway in the procyclic form.

In the bloodstream form mitochondria, the mechanism could be different. Besides two pathways that are described above, an alternate route of import for inner membrane protein is via the carrier import pathway. In this process, the precursor proteins that do not have a N-terminal signal but possess internal signals, such as mitochondrial metabolite carrier proteins, are imported into mitochondria that requires less $\Delta\psi$ and does not depend on ATP hydrolysis in the matrix (Pfanner and Geissler, 2001; Endo et al., 2003; Neupert and Herrmann, 2007). Apparently, TAO does not have the characteristics of this group of proteins. However, a possibility for the presence of the internal import signal(s) can not be excluded.

It is surprising, that the import of COIV into mitochondria is also independent of $\Delta\psi$ in the bloodstream form. We have verified that mitochondria isolated from the bloodstream form by isotonic lysis method possess membrane potential, which is sensitive to valinomycin and CCCP. Furthermore, after additional purification of mitochondria following *in vitro* import, we showed that the precursor proteins entered into mitochondria and not trapped in any membrane bound organelles. Thus, these are unique examples that the same precursor proteins are being imported into mitochondria by different mechanisms in two developmental stages of *T. brucei*.

Although, $\Delta\psi$ is one of the essential constituents for import of mitochondrial proteins in the matrix and the inner membrane, there are few exception have also been demonstrated. Mitochondrial transcription factor Mtf1P in yeast does not depend on either $\Delta\psi$ or matrix ATP for its import (Biswas and Getz 2002). Furthermore, this protein depends on the N-terminal targeting signal for its import, when synthesized on rabbit reticulocyte translation system, but does not depend on this signal while the precursor protein was synthesized in the wheat germ extract, suggesting that the same protein is capable of utilizing targeting signals located at different regions of the protein depending on the cytosolic factor(s) present in the specific translation system (Biswas and Getz, 2004). In addition, *T. brucei* cyt c1 also known to be imported in the procyclic mitochondria in the presence of $\Delta\psi$ inhibitors, whereas, in other system import of cyt c1 is strictly dependent on $\Delta\psi$ (Priest and Hajduk, 2003).

In yeast, when the respiratory chain is damaged by mutation or when cells lose mitochondrial DNA, creating “petite” mutants, mitochondrial inner membrane potential is thought to be

maintained by combining activities of mitochondrial ADP/ATP translocator and matrix localized F1ATPase (Kerscher et al., 2000), which is similar to mitochondria of the bloodstream form of *T. brucei*. It is not clear if the mitochondrial protein import mechanism is different in these mutant yeast cells; however, a few non-essential protein translocators of the mitochondrial outer and inner membrane (Tom and Tim) have been shown to be essential in petite cells such as Tim18 and Tom70 (Kerscher et al., 2000). In addition, the high copy number suppressor mutants of these non essential Toms and Tims are those, that over produce few cytosolic chaperones or proteins involved in translational machinery (Dunn and Jensen, 2003).

The protein translocation machinery in kinetoplastid parasites totally remains unexplored. Homologs for known Tom and Tim proteins are extremely rare in trypanosomatids genome databases. Thus, it can be anticipated that the mechanistic details for import of mitochondrial proteins could be different in these earliest eukaryotes. Here, our results revealed that TAO and COIV are imported into mitochondria in the bloodstream form in a distinct mechanism, which could be different from its mammalian hosts.

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Abbreviations

TAO, Trypanosome alternative oxidase; COIV, Cytochrome oxidase subunit IV; Ndh K, NADH dehydrogenase K; CCCP, Carbonyl cyanide m-chlorophenyl hydrazone; DMSO, Dimethyl sulfoxide; PSORT, Prediction of protein sorting signals and localization sites in amino acid sequences.

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Fig. 1.

In vitro import of TAO in the procyclic mitochondria. The ³⁵S-methionine labeled precursor TAO was synthesized using an *in vitro* transcription/translation system and was incubated with isolated mitochondria from the procyclic form in appropriate buffer for 30 min. (A) After import the samples were divided into two parts; one part was treated with proteinase K and the other part was left untreated as indicated by '+' and '-' sign. Mitochondria were re-isolated and proteins were analyzed by SDS-PAGE and autoradiography. Ten percent of radiolabeled proteins (Input 10%) used in the import reaction was also analyzed. (B) Mitochondria were pretreated with valinomycin and import reaction was performed as described above. (C) Mitochondria were pretreated with trypsin, as indicated on the top of autoradiogram, to remove

proteins from the outer surface. In some experiments the post-import proteinase K treatment was performed in the presence of Triton X-100 (1 %) (TX-100). A 10% of radiolabeled proteins (Input 10%) used in the import reaction was also analyzed. (D) After import of radiolabeled TAO mitochondria were extracted with Na_2CO_3 . The supernatant and the pellet fractions were analyzed as described. Each blot was immunodecorated with mHsp70 antibody to verify that PK digestion did not damage the mitochondrial membrane. The precursor and the mature form of TAO were indicated by p and m.

Fig. 2.

In vitro import of TAO in the mitochondria of the bloodstream form. The labeled precursor TAO was incubated with isolated mitochondria from the bloodstream form in appropriate buffer for 30 min. (A) Incubation temperature was varied from 25-37 °C. After import mitochondria were washed and proteins were analyzed by SDS-PAGE and autoradiography. The precursor and the mature form of TAO were indicated by p and m. (B) The intensity of the matured TAO protein was quantitated using imaging densitometer as described in the materials and methods and plotted against incubation temperature. (C) *In vitro* import of radiolabeled TAO was performed as stated before. The post-import Proteinase K-digestion was done in the presence (+) and absence (-) of Triton X-100 (TX-100) as indicated on the top of

the autoradiogram. (D) Na_2CO_3 extraction of mitochondria was performed after import of TAO as described in the previous figure. A 10% of radiolabeled proteins (Input 10%) used in the import reaction was analyzed. The precursor and the mature form of TAO were indicated by p and m as described before.

Fig.3.

Isolated mitochondria from both the procyclic and the bloodstream forms of *T. brucei* possess a membrane potential and a double membrane. (A) Isolated mitochondria from the procyclic and bloodstream forms were incubated in the presence and absence of membrane potential inhibitors valinomycin (0.5 μ M) and CCCP (50 μ M) for 15 min on ice. Following incubation, mitochondria were stained with MitoTracker green and images were taken by confocal microscopy as described in the materials and methods. MitoTracker green was excited with a 488 nm argon laser, and the emitted light was collected using a 505-550 nm bandpass filter. All images were collected with exactly the same detector gain, laser strength, and number of scans in order for the data to be examined for differences in fluorescence intensity. The phase

contrast pictures (DIC) of the procyclic and bloodstream form mitochondria were shown. (B) The kinetic measurement of the fluorescence intensity of DisC3 was performed in Fluoromax-3 spectrophotometer. Mitochondria isolated from the bloodstream and the procyclic forms were added to the fluorescent dye DisC3 in the import buffer at indicated time. Valinomycin (0.5 μ M) was added to the mixture as indicated to dissipate membrane potential. Time of addition of Mitochondria (Mito) and valinomycin (Val) was indicated by arrows. (C) Electron micrographs of the thin section of the purified mitochondria from the bloodstream and the procyclic forms. The sections reveal the double membranes. A majority of the vesicle in fields showed similar structure. Among these some possess kinetoplast DNA (not shown). Magnification: 75,000 times.

Fig. 4. Differential requirements of membrane potential for TAO and COIV import in the mitochondria of the procyclic and bloodstream forms. (A) Mitochondria were treated with different concentrations (0-150 μM) of valinomycin and CCCP before addition of the radiolabeled precursor TAO. (B) Import was also performed using radiolabeled precursor COIV in the presence (+) and absence (-) of valinomycin and CCCP. Import reactions were performed at 25 °C for the procyclic and at 37 °C for the bloodstream form mitochondria for 30 min and imported proteins were analyzed by SDS-PAGE and autoradiography as described previously. A 10% of radiolabeled proteins (Input 10%) used in the import reaction was loaded

on the same gel. The precursor and the mature form of TAO and COIV were indicated by p and m.

Fig.5.

Labeled TAO protein is imported into mitochondrial vesicle. To verify that the labeled precursor TAO protein is indeed imported into mitochondria isolated from the bloodstream form, the import reaction was scaled up to a final volume of 500 μ l. Import was conducted for 30 min in standard import buffer using crude mitochondrial preparation obtained by differential centrifugation. After that, mitochondria were washed thoroughly with SME buffer and further purified by percol gradient centrifugation as described in the materials and methods. The Top, middle and the bottom layer of the gradient were collected, the membrane was pelleted by centrifugation after dilute with 9 volumes of SME and analyzed for the presence of radiolabeled TAO protein by autoradiography as described previously. The same blot was probed sequentially with TAO and tubulin antibodies to see the presence of endogenous proteins.

Fig. 6.

Import kinetics of TAO and COIV into the mitochondria of the procyclic and the bloodstream form. The ^{35}S -methionine labeled precursor TAO (A) and COIV (B) was incubated with isolated mitochondria from the procyclic and the bloodstream forms at 25 °C and 37 °C, respectively in the import buffer. At different time points equal amounts of samples were collected, mitochondria were re-isolated by centrifugation, washed twice with SME buffer and proteins were analyzed by SDS-PAGE and autoradiography. A fraction of the radiolabeled protein TAO and COIV were also analyzed and indicated as input. The precursor and the mature form of TAO and COIV were indicated by p and m. (C and D) The imported protein bands were quantitated by densitometric scanning (Bio-RAD Model GS-700) using Quantity One software program and were plotted versus times. Results are presented from three independent experiments.

Fig. 7. Requirements of ATP for TAO and COIV import in the mitochondria of the procyclic and bloodstream forms. Standard import reaction was carried out in a 100 μ l final volume, using 100 μ g mitochondrial proteins and 10 μ l of labeled precursor TAO (A & B) and COIV (D) synthesized *in vitro* in the rabbit reticulocyte system. Pretreatment of mitochondria with specific inhibitors oligomycin (30 μ M), atractyloside (400 μ M), were indicated by '+' and '-' for the respective reagent. Addition and depletion of ATP and ATP re-generation system from import reaction were represented by '+' and '-' respectively. Treatment of the reticulocyte lysate with apyrase was performed as described in the materials and methods and indicated similarly as '+' and '-' sign. After import, mitochondria were reisolated, washed, and the

radiolabeled protein was analyzed by SDS-PAGE and autoradiography as described above. A 10% of radiolabeled proteins (Input 10%) used in the import reaction was loaded on the same gel. The precursor and the mature form of TAO were indicated by p and m. (C) ATP concentration was measured using a luciferase based ATP assay kit as described in the materials and methods. Procyclic and bloodstream mitochondria were incubated in the presence and absence of oligomycin (30 μ M) before ATP assay. ATP concentration after treatment of mitochondria with oligomycin was presented as the percent of untreated control.