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In vitro import of proteins into mitochondria of *Trypanosoma brucei* and *Leishmania tarentolae*

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SUMMARY

In eukaryotic evolution, the earliest branch of organisms to have mitochondria are the trypanosomatids. Their mitochondrial biogenesis not only includes import of most proteins, but also, unlike in other organisms, import of the whole set of tRNAs. In order to investigate these processes, we devised novel procedures for the isolation of mitochondria from two trypanosomatid species: *Trypanosoma brucei* and *Leishmania tarentolae*. Isotonic cell lysis followed by equilibrium density centrifugation in Nycodenz gradients yielded mitochondrial fractions exhibiting a membrane potential. Furthermore, we have used these fractions to reconstitute import of mitochondrial matrix proteins in vitro. Energy-dependent uptake of an artificial precursor protein, containing a trypanosomal presequence attached to mouse dihydrofolate reductase and of yeast mitochondrial alcohol dehydrogenase could be demonstrated. The presequences of both proteins were processed in *T. brucei* whereas only the trypanosomal one was cleaved in *L. tarentolae*. Trypsin pretreatment abolished the ability of the mitochondria to import proteins, indicating the involvement of proteinaceous components at the surface of mitochondria.

Key words: Mitochondrial biogenesis, Protein import, *Kinetoplastidae*

INTRODUCTION

The single mitochondrion of trypanosomatids exhibits a number of unique features compared to mitochondria from other organisms. They possess a bipartite genome organized in a network of topologically interlocked maxi- and minicircles (Borst, 1991). Transcripts originating from cryptogenes on the maxicircle DNA have to undergo RNA editing in order to yield translatable mRNAs (Simpson, 1990). Furthermore, it has been shown in at least one trypanosomatid, *T. brucei*, that the mitochondrial metabolism is different from that in yeast and in mammalian cells. The Krebs cycle enzyme citrate synthase appears to be missing (Fairlamb and Opperdoes, 1986), and the electron transport chain shows a different sensitivity to some respiration inhibitors from its counterpart in mammalian cells (Turrens, 1989). Also, unlike in most other organisms, no tRNA genes have been found in the mitochondrial genome of trypanosomatids. Instead, the mitochondrial tRNAs were found to be encoded on the nuclear genome (Hancock and Hajduk, 1990; Mottram et al., 1991; Simpson et al., 1989). Mitochondrial biogenesis in trypanosomes therefore involves not only import of proteins, as in all other eukaryotes, but also import of the whole set of mitochondrial tRNAs (Chen et al., 1994; Schneider et al., 1994).

Isolation procedures based on hypotonic cell lysis which yield highly pure mitochondrial fractions from *T. brucei* (Harris et al., 1990) and *L. tarentolae* (Braly et al., 1974) have been described. However, no membrane potential could be detected in those preparations. The study of energy-linked mitochondrial functions in trypanosomatids has therefore been restricted to digitonin-permeabilized cells (Turrens, 1989; Vercesi et al., 1991). Protein import into the matrix of mitochondria depends on a membrane potential (Gasser et al., 1982); consequently organelles isolated by hypotonic lysis were not able to translocate proteins across the mitochondrial inner membrane. Research on this important aspect of mitochondrial biogenesis has therefore been limited to in vivo systems (Häusler et al., 1996).

Here we present a novel purification procedure for mitochondria of *T. brucei* and *L. tarentolae* that preserves the mitochondrial membrane potential. In addition, we devised protocols to study mitochondrial protein import in vitro. Precursor proteins bearing either a homologous or a yeast mitochondrial presequence could be translocated across the mitochondrial inner membranes of *T. brucei* and *L. tarentolae*.

MATERIALS AND METHODS

Cells

Procyclic *T. brucei*, stock 427, were grown at 27°C in SDM-79 medium supplemented with 5% fetal bovine serum. Cells were

harvested at late log phase corresponding to 1.5×10^7-2.5×10^7 cells/ml. *L. tarentolae* (UC strain) was grown at 27°C in Difco brain heart infusion medium containing 10 μg/ml hemin to late log phase (0.5×10^8-1.5×10^8) and used immediately. Cells from both organisms were washed once in cold 20 mM sodium phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose.

Isolation of the mitochondrial fraction of *T. brucei*

For each preparation 2×10^{10} cells were used. All steps were performed at 0-4°C. Washed *T. brucei* were resuspended in SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.9, and 2 mM EDTA) at a concentration of 2×10^9 cells/ml and disrupted by intracytoplasmic cavitation of nitrogen gas (Wallach and Kamat, 1964). The cell suspension was placed in a prechilled cavitation chamber and 50 bar of pressure were applied for 45 minutes. Finally, the pressure was abruptly released yielding a cell lysate. The lysate was centrifuged at 15,800 ***g***, the pellet (Fig. 1, P1) was resuspended in an equal volume of SoTE and, after addition of $MgCl_2$ to 6 mM, the sample was treated with DNase I (50 μg/ml). After 15 minutes at 0-4°C the treatment was stopped by the addition of EDTA to 10 mM, the sample was centrifuged under identical conditions and the pellet (Fig. 1, P2) was resuspended in approximately 2 ml of SoTE containing 50% Nycodenz (Nycomed Pharma, distributed by Life Technologies, Switzerland). Samples of 0.5 ml each, corresponding to 5×10^9 cell-equivalents, of that suspension were loaded below a 15%/18.8%/21.7%/25% (v/v)-Nycodenz step gradient containing 1× SoTE. Four gradients were prepared (in ultra clear centrifuge tubes, 14 mm × 98 mm) containing 2.5 ml of each Nycodenz solution and used immediately. After spinning the gradients for 40 minutes at 293,000 ***g*** (TST41.14, 40,000 rpm) the band corresponding to the mitochondrial fraction accumulated at the 18.8%/21.7% Nycodenz interface. The mitochondrial fraction was collected, washed once with an excess of SoTE (32,500 ***g***, 10 minutes) to remove the Nycodenz and finally resupended in a small volume of SoTE (protein concentration approximately 20 mg/ml). Mitochondrial vesicles were either used directly or after addition of fatty-acid-free bovine serum albumin to 10 mg/ml, frozen in liquid nitrogen and stored at −70°C.

Isolation of the mitochondrial fraction of *L. tarentolae*

Except for the following modifications, the mitochondrial isolation procedure for *L. tarentolae* is identical to *T. brucei*. For each preparation 4×10^{10} cells were used. Nitrogen cavitation was performed at 4×10^9 cells/ml at 50 bar for 1 hour. After the DNase I digestion the lysate was resuspended in 3 ml SoTE containing 50% Nycodenz; 1×10^9 cell equivalents (0.75 ml) were loaded onto each 21.7%/25%/28.3/31.6% (v/v) Nycodenz step gradient and the mitochondrial fraction was collected at the 25%/28.3% interface.

Measurement of membrane potential

The potential measurements were performed at 25°C with the potential-sensitive dye (3,3)-dipropylthiocarbocyanine iodide (diS-C_3-(5) (Hwang et al., 1989; Sims et al., 1974), Molecular Probes, Inc., Junction City, OR) on a Jasco FP-777 spectrofluorometer. Excitation was at 620 nm, emission at 670 nm. A 2 mM stock of diS-C_3-(5) in ethanol was diluted 400-fold into import buffer (20 mM HEPES-KOH, pH 7.4, 0.6 M sorbitol, 25 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM KH_2PO_4, 1 mg/ml fatty-acid-free bovine serum albumin) containing 5 mM NADH, 5 mM succinate and 1 mM ATP and constantly stirred. Measurements were initiated by adding 100 μg proteins of the mitochondrial fractions. Finally, mitochondria were uncoupled by adding valinomycin to 2.5 μM.

Protein import into isolated mitochondria of *T. brucei* and *L. tarentolae*

Import conditions for *T. brucei* and *L. tarentolae* mitochondria were identical. A fusion protein containing the amino-terminal 14 amino acids of the trypanosomal mitochondrial dihydrolipoamide dehydrogenase (LDH) (Else et al., 1993) attached to cytosolic mouse dihydrofolate reductase (DHFR) and the precursor of mitochondrial alcohol dehydrogenase III (ADHIII) from *S. cerevisiae* (Young and Pilgrim, 1985) were used as import substrates. Radioactive precursors of the fusion protein LDH-DHFR and ADHIII were synthesized in vitro from mRNAs in a reticulocyte lysate containing [^{35}S]methionine. The mRNAs were obtained by in vitro transcription of vectors containing the corresponding genes, using the SP6 promoter for ADHIII and the T7 promoter for LDH-DHFR. After translation, ribosomes were removed by centrifugation (10 minutes, 100,000 ***g***).

Import was done in import buffer (see above) supplemented with 5 mM NADH, 5 mM succinate, 0.5 mM methionine, 1 mM ATP, and an ATP-regenerating system consisting of 6 mM creatine phosphate and 80 μg/ml creatine phosphokinase. Standard import conditions were as follows: 15 or 10 μl of in vitro translated LDH-DHFR or ADHIII precursors, respectively, were added to 100 μl of supplemented import buffer. Import was initiated by adding 100 μg proteins of the mitochondrial fraction (approximately 5 μl) and incubating the reaction at 23°C for 20 minutes. In the indicated samples 1 μl of valinomycin stock (0.2 mM) and 1 μl of carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone stock (FCCP)(5 mM) were added to destroy the membrane potential. In some samples, which are marked −ATP, ATP and the regenerating system were omitted and ATP was depleted from the in vitro translate by apyrase and from the mitochondria using oligomycin, efrapeptin and carboxyatractyloside as described (Wachter et al., 1994). After import, most samples were treated with proteinase K (200 μg/ml). As a control for the efficiency of the protease, the digestion was also performed in the presence of 0.5% Triton X-100. After 20 minutes at 0-4°C the protease digestion was stopped by the addition of PMSF to 1 mM. Mitochondria from all import reactions were reisolated by centrifugation (5,500 ***g***, 5 minutes, 4°C) and resuspended in SDS sample buffer. Detergent-treated samples could not be reisolated but were directly processed for gel-electrophoresis. As an additional control, both import of LDH-DHFR and ADHIII into mitochondria from *T. brucei* and *L. tarentolae* was compared to import of the same precursors into mitochondria of *S. cerevisiae* under identical conditions; 50 μg of Nycodenz-purified yeast mitochondria (Glick and Pon, 1995) were used in this case.

Import reactions were analyzed on 14% (LDH-DHFR) or 10% (ADHIII) polyacrylamide SDS gels, respectively. The gels were boiled in 5% TCA, neutralized in 1 M Tris, incubated for 30 minutes in 1 M sodium salicylate, dried and exposed on film for 1 to 7 days.

Miscellaneous

Immunoblots and fluorograms were quantified using a PhosphorImager SF (Molecular Dynamics). Protein concentrations were measured using the BCA procedure (Pierce Chemical Co.). Samples for transmission electron microscopy were fixed in SoTE containing 2.5% glutaraldehyde and processed as described (Lucocq et al., 1987).

RESULTS

Purification of energized mitochondria

Existing mitochondrial isolation procedures for *T. brucei* and *L. tarentolae* rely on hypotonic cell lysis and subsequent Percoll or Renografin gradient centrifugations (Braly et al., 1974; Harris et al., 1990). Those preparations yield highly purified mitochondrial vesicles suitable for biochemical studies. In *T. brucei* the isolated mitochondria still exhibit transcription as well as RNA editing activity (Harris et al., 1990). However, mitochondria isolated by the 'hypotonic lysis protocols' from either organism were not able to import precursor proteins. This is most likely due to the fact that no

membrane potential, which is an absolute requirement for import of mitochondrial matrix proteins, could be detected in those fractions (not shown). Our approach, therefore, was to design a mitochondrial isolation procedure for both *T. brucei* and *L. tarentolae* yielding energized mitochondria capable of protein import. When hypotonic cell lysis is used, it is impossible to avoid potentially damaging exposure of mitochondria to the hypotonic environment. As an alternative we used nitrogen cavitation which allowed efficient breakage of the cells under isotonic conditions in an inert atmosphere without danger of local heating (Wallach and Kamat, 1964). *T. brucei* cells were efficiently lysed (>95%), whereas lysis of *L. tarentolae* was less efficient (>90%) as estimated microscopically. Furthermore, 0.25 M sucrose used as an osmotic balancer in the original protocols was replaced by 0.6 M sorbitol because the latter provided more gentle conditions for mitochondria. After a DNase I digestion, the crude organellar fractions were resolved on Nycodenz step gradients. Mitochondria from *T. brucei* are less dense than mitochondria from *L. tarentolae*, therefore the range of percentages of Nycodenz had to be optimized for each organism. A 15%-25% Nycodenz step gradient (see Material and Methods) was found to be optimal to separate *T. brucei* organelles. The top of the gradient contained intact cells only, many of which were still alive. The band at the 15%/18.8% Nycodenz boundary contained mitochondrial vesicles heavily contaminated by intact cells. Most material on the gradient was found at the 18.8%/21.7% Nycodenz interface. This fraction consisted mainly of mitochondrial vesicles, while hardly any whole cells could be detected. Finally, flagella accumulated in the 21.7%/25% Nycodenz region.

The best separation of *L. tarentolae* organelles was achieved on 21.7%-31.6% Nycodenz step gradients (see Materials and Methods). At the top of the gradients intact cells and cell shaped cytoskeleton structures accumulated. The main fraction of the gradient, containing some mitochondrial vesicles and cell shaped debris, was recovered at the 21.7%/25% Nycodenz interface. The band in the 25%/28.3% Nycodenz region was most enriched for mitochondrial vesicles; however, unlike for *T. brucei*, few intact cells were still present in that fraction. Finally, in the most dense fraction (28.3% /31.6% Nycodenz) mainly flagella were detected.

Characterization of mitochondrial fractions

The following criteria were used to characterize the isolated mitochondrial fractions. First, purification factor and yield were determined by immunoblotting of the different fractions (Fig. 1, Table 1) with a mitochondria-specific polyclonal antiserum directed against yeast chaperonin 60, a highly conserved mitochondrial matrix protein (Cheng et al., 1989). In representative experiments the antigen was found to be 3.2-fold enriched (yield 9.6%) in the 18.8%/21.7% Nycodenz gradient fraction of *T. brucei* and 2.9-fold enriched (yield 2.1%) in the corresponding fraction (25%/28.3% Nycodenz) of *L. tarentolae* (Table 1). Some degradation of chaperonin 60 was observed in the mitochondrial fractions as indicated by the double bands observed in the immunoblots (Fig. 1).

Second, isolated mitochondrial fractions were examined under a light microscope. In the *T. brucei* sample vesicles of fairly uniform size were observed. Ethidium bromide fluorescence microscopy (Harris et al., 1990) showed that approximately one third of those vesicles contained DNA. The *L. tarentolae* mitochondrial fraction looked very uniform: large vesicles, some of which contained DNA, and occasionally whole cells were observed. The same samples were also analyzed by transmission electron microscopy. For *T. brucei* a population of vesicles (diameter: 0.9±0.3 μm) containing cristae like structures (Fig. 2A) could be seen. The organelles appeared to be aggregated. It is therefore difficult to discern the double membrane expected for mitochondria, even at high magnifications (Fig. 2A, inset). Thus, it is not possible to assess the extent to which the outer membrane has been disrupted. Fig. 2B shows the corresponding mitochondrial preparation of *L. tarentolae*: a uniform population of cristae-containing double membrane-bound vesicles (diameter: 1.6±0.4 μm) is observed. Outer membranes are intact since they can be traced all around individual organelles (Fig. 2B, inset).

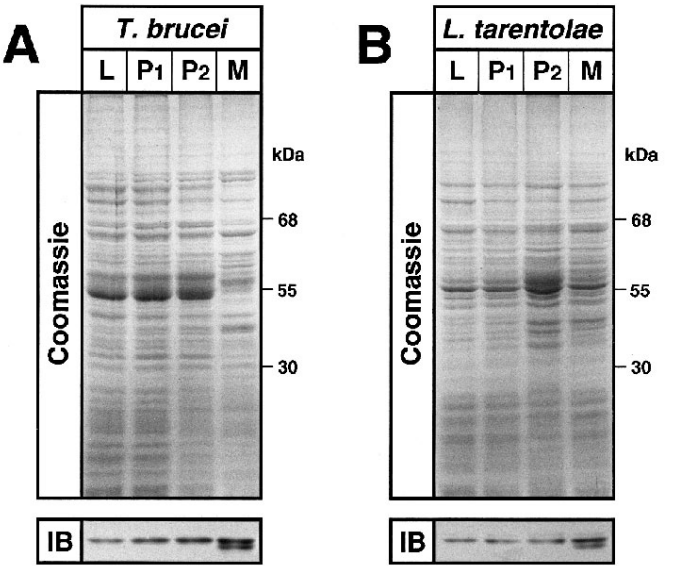

Fig. 1. Isolation of mitochondrial fractions of *T. brucei* (A) and *L. tarentolae* (B). Aliquots (40 μg each) of total cell lysate (L), the organellar pellet after cell lysis (P1), the pellet after DNase I treatment (P2) and of the mitochondrial fraction (M) from the Nycodenz gradients were run on two SDS-10% polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue (upper panel); the other gel was subjected to immunoblotting with an antiserum to yeast chaperonin 60. Signals on the immunoblot were quantified and used to calculate purification factors and yields (Table 1).

Table 1. Isolation of mitochondrial fractions

	Protein (mg)	Yield (%)	Purification (-fold)
T. brucei			
Cell lysate	74	100	1.0
P_1	36.5	79	1.6
P_2	11	25.3	1.7
Mitochondria	2.2	9.6	3.2
L. tarentolae			
Cell lysate	207	100	1.0
P_1	93	49.4	1.1
P_2	26.2	16.5	1.3
Mitochondria	1.5	2.1	2.9

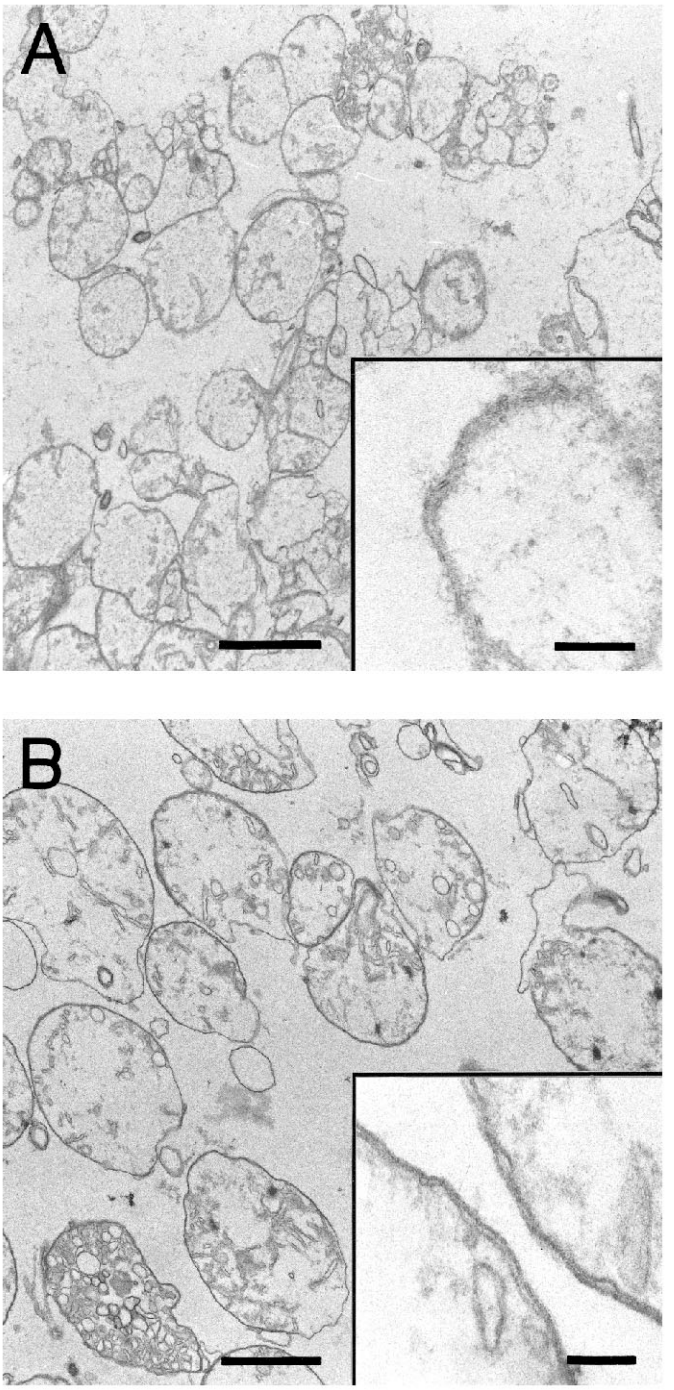

Fig. 2. Electron micrographs of thin sections of *T. brucei* (A) and *L. tarentolae* (B) mitochondrial fractions from the corresponding Nycodenz gradients. High magnification insets reveal double membranes. Bars, 1 μm (insets, 0.1 μm).

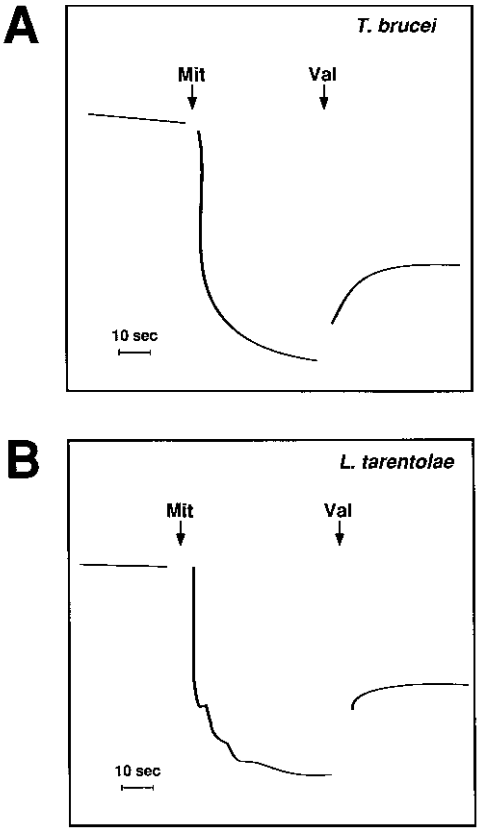

Fig. 3. Fluorescence measurements of membrane potentials generated by *T. brucei* (A) and *L. tarentolae* (B) mitochondrial fractions (Mit). Fluorescence is given in arbitrary units; an increase in potential is represented by a downward deflection. Where indicated, valinomycin (Val) was added to 2.5 μM to destroy the membrane potential.

Finally, the isolated mitochondrial fractions were tested for their ability to generate a membrane potential. This was done using the potential-sensitive dye diS-C_3-(5) (Hwang et al., 1989; Sims et al., 1974). Fig. 3 shows that both samples exhibited a membrane potential as indicated by the valinomycin-sensitive downward deflection of the fluorescence after the addition of mitochondrial vesicles. Further addition of valinomycin did not have any effect (not shown). The membrane potential was still detectable even four hours after isolation, provided that the mitochondria were kept on ice and at a high concentration (approximately 20 mg/ml) in SoTE buffer (see Materials and Methods).

Import of proteins into isolated mitochondria

An electrochemical potential across the inner membrane is a stringent requirement for translocation of proteins into the mitochondrial matrix (Gasser et al., 1982). Our new isolation procedure clearly yields mitochondria with an intact membrane potential. In order to test the import competence of these mitochondria, two protein substates were used which are targeted to the mitochondrial matrix. The first one, LDH-DHFR, contains the amino-terminal 14 amino acids of the dihydrolipoamide dehydrogenase (LDH) of *T. brucei* (Else et al., 1993) fused to dihydrofolate reductase (DHFR). As a second precursor, alcohol dehydrogenase III (ADHIII) of yeast (Young and Pilgrim, 1985) was used. Since mitochondrial protein import in yeast has been characterized in great detail (Hannavy et al., 1993; Segui-Real et al., 1992), it served as a control for the import assays in trypanosomatids. In vitro import was performed for both precursor proteins under identical conditions in all three organisms (Fig. 4). LDH-DHFR was imported and its presequence concomitantly

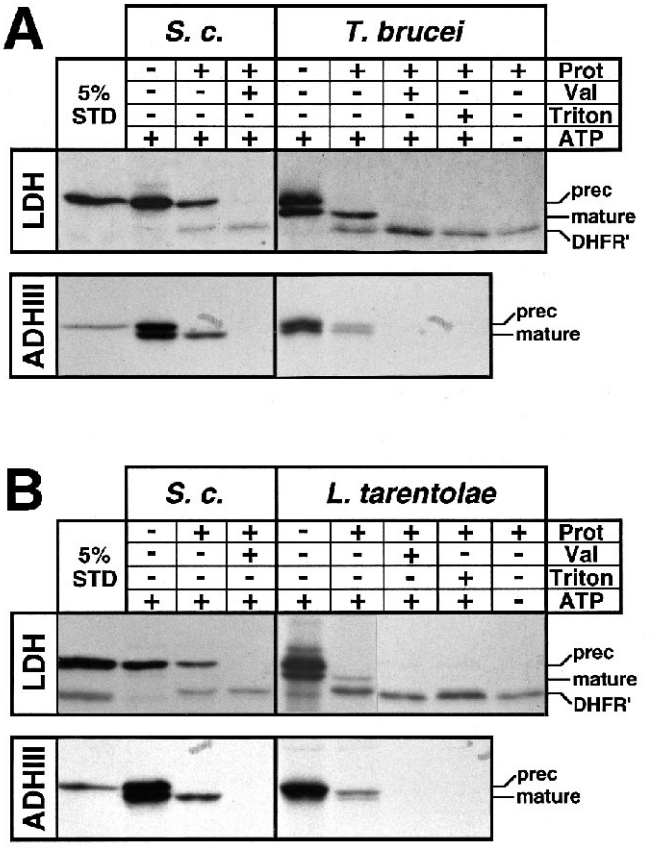

Fig. 4. In vitro import of LDH-DHFR (LDH) and ADHIII into mitochondria of *S. cerevisiae (S. c.), T. brucei* (A) and *L. tarentolae* (B). Import was performed under standard conditions (see Materials and Methods). Where indicated (+) proteinase K (Prot), valinomycin (Val) or Triton X-100 (Triton) were added to the reactions. ATP depletion in the −ATP sample was performed as described (Wachter et al., 1994). The positions of the precursors of LDH-DHFR and ADHIII (prec), their processed mature forms (mature) and the protease resistant fragment of LDH-DHFR (DHFR′) are indicated. 5% STD, 5% of the amount of precursor mixture initially added to each import reaction. In some in vitro translation reactions of LDH-DHFR an additional lower molecular mass band appeared due to a second start site at an internal methionine (B, STD).

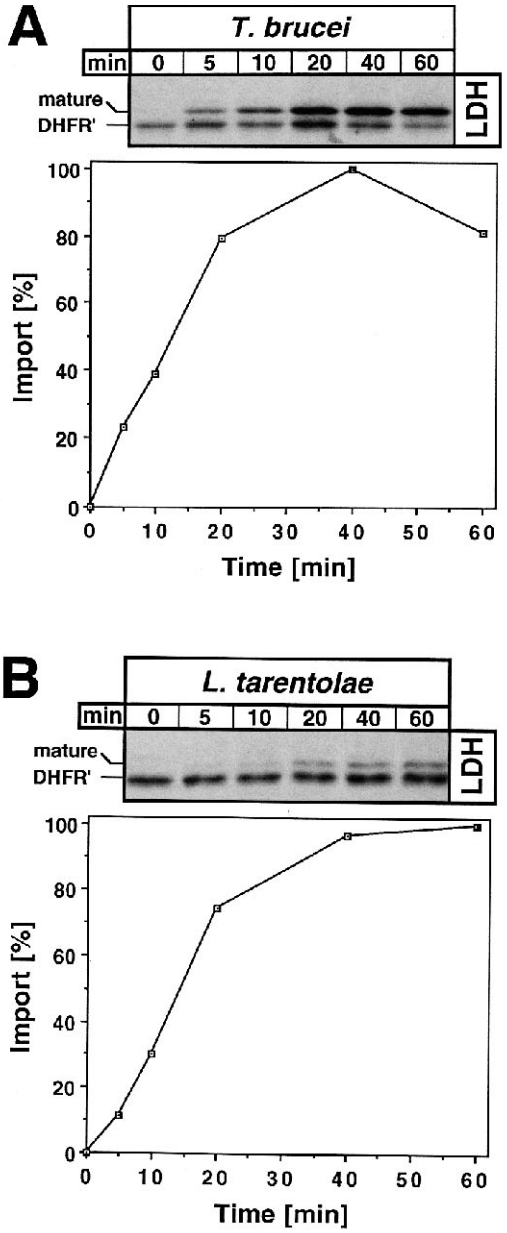

Fig. 5. Time course of LDH-DHFR import at 23°C into mitochondria of *T. brucei* (A) and *L. tarentolae* (B). All reactions were proteinase K treated after import. Photographs of the fluorograms are shown on the upper parts. The relative amounts of protease resistant mature form of LDH-DHFR at the different time points have been used to quantify import (lower parts of figures). Maximal import reached was taken as 100% (100% relative import corresponds to an absolute import efficiency of 1.8% in *T. brucei* and 0.5% in *L. tarentolae*). LDH, mature and DHFR′ as in Fig. 4.

removed in *T. brucei* and *L. tarentolae*. Import was also seen in *S. cerevisiae*; however, no proteolytic processing of the precursor occurred. Also ADHIII was imported in all three organisms. In this case efficient proteolytic removal of the presequence occurred in yeast, partial processing in *T. brucei*, whereas in *L. tarentolae* only residual cleavage was observed. Imported proteins were shown to be resistant to external protease, indicating their intra-mitochondrial localization, and were only digested after disruption of the membranes by detergent. LDH-DHFR got degraded to the protease-resistant DHFR moiety (Fig. 4, DHFR′), whereas ADHIII is completely digested by the protease. The protease resistant DHFR fragment (DHFR′) is expected to be lost during reisolation of mitochondria after the import reaction. Nevertheless, some variable non-specific binding of small amounts of DHFR′ to the mitochondrial fraction was observed. As predicted, translocation of proteins across the inner membrane depends on an electrochemical potential and on ATP as an energy source (Wachter et al., 1994). No import was detected after addition of the ionophores valinomycin and FCCP or in the absence of ATP.

Import efficiencies, defined as the percentage of protease resistant precursor or mature form after standard import reactions, were determined: variable amounts between 0.5-1.8% of LDH-DHFR could be imported into *T. brucei* or *L. tarentolae* mitochondria. Generally, more LDH-DHFR was imported into *T. brucei* than into *L. tarentolae* mitochondria.

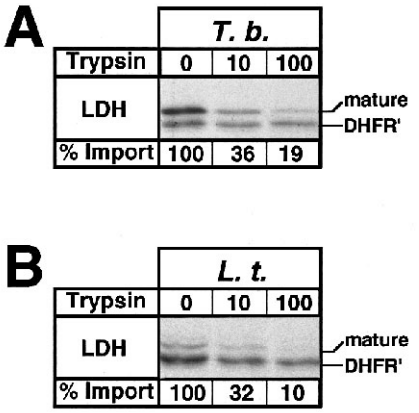

Fig. 6. Trypsin pretreatment of mitochondria abolishes import of LDH-DHFR into mitochondria of *T. brucei* (A) and *L. tarentolae* (B). Pretreatment with trypsin at 10 or 100 μg/ml was performed as described (Hines et al., 1990), except mitochondria were not reisolated after trypsin digestion but directly used for import which was performed for 20 minutes at 23°C for *T. brucei* and for 30 minutes at 27°C for *L. tarentolae*. Trypsin pretreatment was stopped by adding soybean trypsin inhibitor to 1 mg/ml. All samples were treated with proteinase K after import. Relative import efficiencies, defined as the percentage of protease resistant mature form of LDH-DHFR, of trypsin treated samples compared to untreated controls are indicated at the bottom of each panel. LDH, mature and DHFR′ as in Fig. 4.

Using ADHIII better efficiencies of ~5% were reached in both trypanosomatid species. In yeast, import efficiencies were slightly higher: ~2% of LDH-DHFR and ~8% of ADHIII were imported. Generally, these import efficiencies, especially for LDH-DHFR, are low. This may partly be due to the short 9 amino acid-presequence of LDH-DHFR which may not work well in vitro and which might easily be inactivated by contaminating proteases present in the mitochondrial fractions. The presence of proteases is evident from the observation that supernatants of reisolated mitochondria after an import reaction only contained the protease resistant DHFR′ fragment but not the intact precursor of LDH-DHFR (not shown).

Import of LDH-DHFR into *T. brucei* and *L. tarentolae* mitochondria was complete after 40 minutes at 23°C as indicated by time course experiments (Fig. 5). The standard import reactions were incubated for 20 minutes as the kinetics of import were still linear at that time.

Mitochondrial vesicles that had been exposed to low concentrations of trypsin prior to incubation with the in vitro synthesized precursors showed a great reduction (80-90%) of import. This indicates the involvement of proteins on the surface of the mitochondrial vesicles in the import process (Fig. 6).

Altogether these results show that we have established a bona fide in vitro import system for proteins into the mitochondrial matrix of trypanosomatids. The properties of the assay are very similar to those of the well-characterized system in yeast and therefore are believed to reflect the in vivo situation.

DISCUSSION

Trypanosomatid mitochondria are unique in many respects such as genome organisation, RNA editing and tRNA import. Phylogenetic analysis of small subunit ribosomal RNAs shows a very early branching of trypanosomatids from the eukaryotic evolutionary tree, providing an explanation for the unusual properties of the mitochondria of this family of organisms. In fact, trypanosomatids are the oldest distinct eukaryotic lineage that have mitochondria and, thus, they are only distantly related to most other eukaryotes (Sogin et al., 1986).

We have devised a novel procedure to isolate mitochondria from two trypanosomatid species, *T. brucei* and *L. tarentolae*, which yields mitochondrial fractions exhibiting an electrochemical potential across the inner membrane. This isolation protocol provides a new tool for mitochondrial research in trypanosomatids. Studies of energy-linked functions of mitochondria (Fairlamb and Opperdoes, 1986; Turrens, 1989) can now be carried out using isolated organelles instead of intact or permeabilized cells. In addition, the proposed method offers the possibility to study mitochondrial protein import in vitro.

Many features of mitochondrial protein import are conserved in trypanosomatids. Two components of their protein import machinery have been cloned: chaperonin 60 (Giambiagi de Marval et al., 1993) which is involved in refolding of imported proteins, and the putative import motor, mitochondrial heat shock protein 70 (Engman et al., 1989). Both are highly homologous to the corresponding counterparts in other species. Nuclear genes encoding a number of mitochondrial proteins have been cloned from trypanosomatids. Often, the presence of an amphipatic presequence could be predicted, but in only few instances has the cleavage of the presequence been confirmed and in no case has the presequence been functionally tested. The putative presequences that are absent from the mature proteins are sometimes exceptionally short - only 8 or 9 amino acids (Clayton et al., 1995). In contrast to the general matrix import pathway, intramitochondrial sorting of proteins might not be conserved in trypanosomatids: cytochrome c_1, which follows a complex import route including two proteolytic processing steps in yeast, lacks a cleavable presequence in the trypanosomatid *Crithidia fasciculata* (Priest et al., 1993).

We have also established an in vitro import system for proteins into mitochondria of *T. brucei* and *L. tarentolae*. Two import substrates were used, LDH-DHFR carrying a homologous trypanosomal 9 amino acid presequence and ADHIII carrying a heterologous yeast 27 amino acid presequence were used. Both proteins were imported into mitochondria from both organisms. The properties of the in vitro system were identical to those of protein import into isolated yeast mitochondria. In all cases, the substrate proteins were translocated across the mitochondrial membranes as assessed by protease treatment. Import was dependent on proteinaceous components on the surface of mitochondria and was shown to require two energy sources: an electrochemical potential across the inner membrane and ATP. Generally, the precursors were cleaved to their mature forms by the corresponding mitochondrial matrix proteases. However, the trypanosomal presequence of LDH was not cleaved by the yeast matrix protease whereas the yeast presequence of ADHIII was very inefficiently cleaved by the leishmanial enzyme, indicating somewhat different substrate preferences of the matrix proteases in the three species.

In summary, our data suggest that mitochondrial import of matrix proteins is a highly conserved process in all eukaryotes.

In vitro import of proteins exhibited identical requirements for mitochondria of yeast and trypanosomatids, two groups of organisms which are only very distantly related (Sogin et al., 1986). Mitochondria of *T. brucei* and *L. tarentolae* import the whole set of their mitochondrial tRNAs (Hancock and Hajduk, 1990; Mottram et al., 1991; Simpson et al., 1989). It has been shown in *S. cerevisiae* that mitochondrial import of $tRNA^{Lys}$, the single imported tRNA, is mediated by the precursor of a mitochondrial aminoacyl tRNA synthetase (Tarassov et al., 1995). If the same mechanism applies to trypanosomatid mitochondria, import of proteins into isolated mitochondria of *T. brucei* and *L. tarentolae* provides a first step towards an in vitro import system for tRNAs.

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