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Unfolding and refolding of a purified precursor protein during import into isolated mitochondria

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A purified mitochondrial precursor protein unfolds to a protease-sensitive conformation at the surface of isolated mitochondria before being imported into the organelles. This unfolding is stimulated by a potential across the mitochondrial inner membrane, but does not require ATP. In contrast, import of the surface-bound unfolded precursor requires ATP, but no potential; it is accompanied by a refolding inside the mitochondria.

Key words: yeast mitochondria/protein import/unfolding/ATP/refolding

Introduction

In every cell, many proteins have to be translocated across one or more membranes in order to reach the compartment in which they are active. Many of these transport events, including the import of proteins into mitochondria, can take place after the polypeptide chain of the translocated protein has been completely synthesized (Wickner and Lodish, 1985; Schatz, 1986). What happens to the conformation of a protein as it is being transported across a membrane? Here we present evidence that a purified mitochondrial precursor protein first unfolds on the mitochondrial suface and then, after translocation, refolds inside the mitochondria.

The mitochondrial precursor is a purified fusion protein consisting of the first 22 amino acids of the presequence of cytochrome oxidase subunit IV (an imported mitochondrial protein) fused to the cytosolic enzyme, mouse dihydrofolate reductase (DHFR). This fusion protein behaves like an authentic mitochondrial precursor protein, both *in vivo* and *in vitro* (Hurt *et al.*, 1984, 1985). It also catalyzes the reduction of dihydrofolate by NADPH with properties very similar to those of authentic mouse DHFR (Eilers and Schatz, 1986; Endo and Schatz, 1988), suggesting that the conformation of the DHFR moiety in the fusion protein is very similar to that of authentic DHFR. We reported previously (Eilers and Schatz, 1986) that binding of the specific DHFR ligand, methotrexate, to the purified fusion protein stabilizes the DHFR moiety and blocks transport of the precursor protein into mitochondria. This observation provided evidence that some unfolding is necessary for translocation to occur. However, it was left open as to whether unfolding occurred outside or inside the mitochondrial membrane and whether or not it required ATP or an energized inner membrane. Also, the proposed unfolded state had not been demonstrated directly. The present study has attempted to answer these questions.

Results

Import of the urea-denatured protein

In the present work, we denatured the precursor protein by dialyzing it overnight against 8 M urea and then quickly diluting it directly into the import reaction containing isolated yeast mitochondria. The resulting final urea concentration during import (0.6 M) did not affect import of the native precursor (Figure 1).

Import of the urea-denatured precursor protein was similar to that of the native precursor in two aspects: it required an energized inner membrane (Figure 1), and it required the presequence, as denatured authentic mouse DHFR was not imported (data not shown). On the other hand, import of the denatured precursor differed from that of the native precursor in three ways. First, it was unaffected by 150 nM methotrexate, which blocks import of the native precursor (Figure 1). Thus, the protein molecules that entered mitochondria had not refolded into a native conformation before being imported. Second, import of the urea-denatured precursor at 25°C was more efficient and at least 10-fold faster than that of the native protein (Figure 2). Third, import of the urea-denatured precursor could occur at much lower temperatures than that of the native protein; for example, at 6°C, the denatured precursor was still imported quite rapidly whereas the native protein was not imported (Figure 2). A slow import of the denatured protein was detectable even at 0°C (not shown).

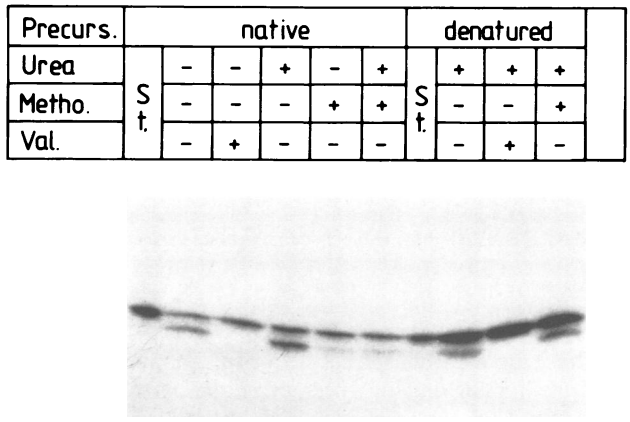

Fig. 1. Methotrexate blocks import of the native, but not of the urea-denatured precursor. Mitochondria (200 μg protein) were incubated with native or with urea-denatured precursor (300 ng; 3×10^8 c.p.m./mg) for either 10 min at 30°C (native precursor) or 1.5 min at 6°C (denatured precursor), respectively. Where indicated, urea (0.6 M final concentration), valinomycin (Val., 5 μg/ml) or methotrexate (Metho., 150 nM) were added to the incubations. The lanes marked St. contained 20% of the amount of precursor added to the mitochondria. The native a pCOX IV-DHFR fusion protein (Eilers and Schatz, 1986) was denatured by dialysis against 8 M urea, 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 250 mM KCl, 1 mM DTT. Isolation of mitochondria and import assays were as described (Gasser *et al.*, 1982).

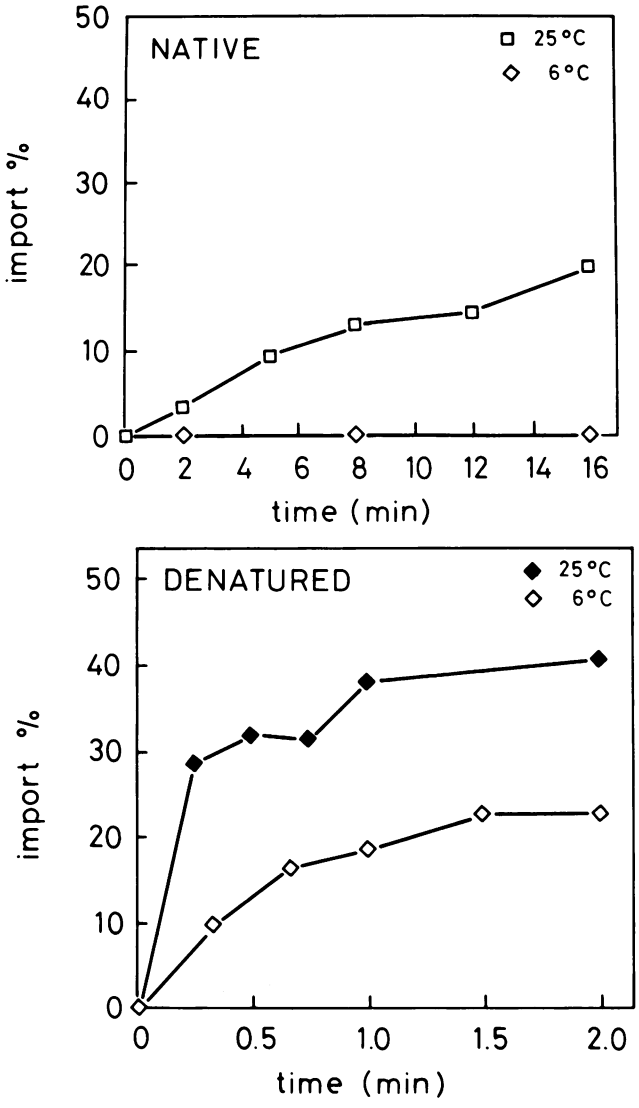

Fig. 2. Import of the denatured precursor is much faster and can occur at lower temperature than that of native precursor. Isolated mitochondria (200 μg protein) were allowed to import the native precursor (300 ng; 5 × 10^8 c.p.m./mg) in the presence of 0.6 M urea (**upper panel**) or the denatured precursor (240 ng; same specific activity) (**lower panel**), either at 25°C or at 6°C. After the indicated times, import was stopped by the addition of antimycin A (final concentration 5 μg/ml), efrapeptin (2.5 μg/ml), valinomycin (5 μg/ml) and dinitrophenol (1 mM). Mitochondria were re-isolated and analyzed for imported protein as above. After gel electrophoresis and fluorography, the amount of 'mature' form was quantified by scanning the autoradiogram.

Thus, the urea-denatured precursor circumvents at least one rate-limiting and highly temperature-sensitive step which is required for the *in vitro* import of the native precursor. We speculated that this step is the unfolding of the native precursor.

An unfolded intermediate in the import of the native precursor

In order to test whether isolated mitochondria unfold the native precursor before importing it, yeast mitochondria were depleted of ATP and then incubated with native precursor in the presence of an ATP trap for 10 min at 30°C. As ATP is necessary for protein import into mitochondria (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Chen and Douglas,

Prot.	−					+				
Chase	−	+				−	+			
ATP	−	−	+	+	+	−	−	+	+	+
Metho.	−	−	−	+	−	−	−	−	+	−
Val.	−	−	−	−	+	−	−	−	−	+

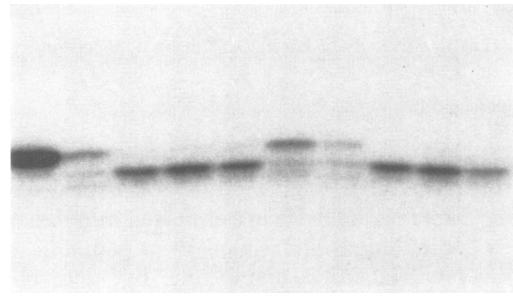

Fig. 3. Precursor accumulated on the mitochondria surface in the absence of ATP can be chased into mitochondria in the presence of methotrexate. Mitochondria (50 μg protein) were incubated with native precursor (450 ng; 7 × 10^7 c.p.m./mg) in the presence of 12.5 units/ml of potato apyrase (Pfanner and Neupert, 1986) and 2.5 μg/ml efrapeptin. After incubation for 10 min at 30°C, they were re-isolated and either left on ice ('− Chase') or re-incubated for additional 10 min at 30°C ('+ Chase'). Where indicated, an ATP-regenerating system (1 mM ATP, 9 mM creatine phosphate, 2.8 U creatine kinase), methotrexate (Metho., 150 nM) or valinomycin (Val., 5 μg/ml) were added to the chase medium. Mitochondria were isolated again and either taken up in SDS−gel electrophoresis sample buffer ('− Prot') or treated (Eilers and Schatz, 1986) with proteinase K ('+ Prot') before gel electrophoresis. The amount of citrate synthase in the samples was determined by immune blotting (Haid and Suissa, 1983) to measure the recovery of mitochondrial protein. Recovery was ~50%.

1987), no import occurred and only the uncleaved precursor was recoverd with the mitochondria upon centrifugation (Figure 3). This precursor was largely accessible to externally added protease, suggesting that it was bound to the mitochondrial surface.

The bound precursor appeared to be a true import intermediate. First, it was imported and cleaved when the mitochondria were resuspended in an ATP-containing buffer. Second, this import was faster and more efficient than that of the native precursor; by correcting for mitochondrial losses during re-isolation and re-incubation we calculated that 50% or more of the bound precursor could be chased into a cleaved, internalized form (Figure 3). This high efficiency of chase also rules out the possibility that the imported, mature form derives from the small amount of protease-protected precursor observed after the incubation in the absence of ATP. Third, the chase was unaffected by 150 nM methotrexate which strongly inhibits import of native precursor. As the bound intermediate was accessible to externally-added protease, lack of inhibition by methotrexate probably did not reflect inaccessibility to the inhibitor. More probably, it reflected a change in the conformation of the precursor which rendered it unable to bind the inhibitor. Fourth, formation of the bound intermediate was strongly dependent on an energized inner membrane; when the potential across the mitochondrial inner membrane was collapsed with antimycin A, binding of precursor to the mito-

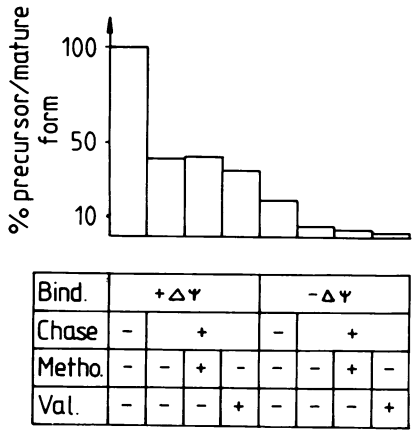

Fig. 4. An energized inner membrane is required to accumulate a surface-bound, chaseable intermediate. Mitochondria (50 μg protein) were incubated with native precursor (300 ng) in the presence of 12.5 U/ml potato apyrase, efrapeptin (2.5 μg/ml) and 1 μg/ml antimycin A and either with ('+ Δ ψ') or without ('− Δ ψ') 0.125 mM *N,N,N',N'*-tetramethylphenylenediamine and 5 mM K-ascorbate, pH 7.4. After incubation for 10 min at 30°C they were re-isolated and either left on ice ('− Chase') or reincubated ('+ Chase') in fresh import buffer containing antimycin A, efrapeptin, ascorbate, TMPD and an ATP-regenerating system (see Figure 3). Where indicated, valinomycin (Val., 10 μg/ml) or methotrexate (Metho., 150 nM) were added. After incubation for an additional 5 min at 30°C the mitochondria were re-isolated and analyzed by SDS−gel electrophoresis and fluorography. The amount of precursor after the binding reactions, or that of mature form after the chase, was quantified by scanning. As regards the fluorogram, the amounts shown for the chase are corrected for a 50% recovery of mitochondria (Figure 3) and for a small amount (<10% of chase values) of mature form generated under ATP-depleted conditions. The amount of precursor accumulated under ATP-depleted conditions in the presence of a membrane potential is arbitrarily set to 100%.

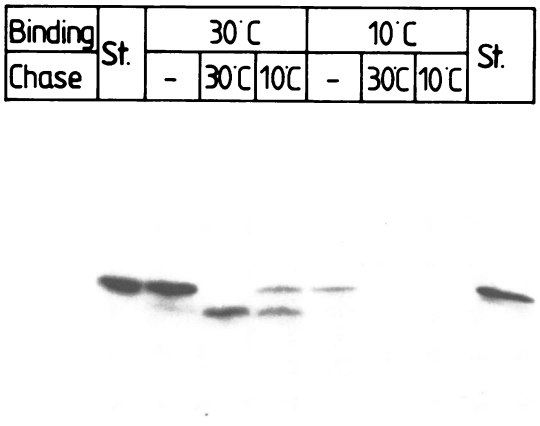

Fig. 5. Precursor accumulated on the mitochondrial surface in the absence of ATP can be chased into mitochondria at low temperature. Mitochondria (50 μg protein) were incubated for 10 min with native precursor (450 ng; 7×10^7 c.p.m./mg) under ATP-depleted conditions ('Binding') either at 30°C (left half) or at 10°C (right half). They were re-isolated and incubated in fresh import buffer containing 150 mM methotrexate and an ATP regenerating system for an additional 10 min at the indicated temperatures ('Chase'). Re-isolation and SDS−gel electrophoresis were carried out as above. St., 10% of the amount of precursor added to mitochondria.

Fig. 6. Precursor accumulated on the mitochondrial surface lacks a protease-resistant DHFR moiety. Mitochondria (50 μg protein) were incubated with native precursor (450 ng; 7×10^7 c.p.m./mg) under ATP-depleted conditions for 10 min at 30°C. They were then re-isolated and lysed in 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 0.5% octyl-polyoxyethylene (octyl-POE, a gift of Boehringer Mannheim, Biocenter). The lysate was incubated with the indicated amounts of trypsin (μg/ml) for 10 min at 0°C and the protease was then blocked by the addition of 1 mM phenylmethyl sulfonyl fluoride (PMSF) (**left panel,** 'Intermediate'). As a control, native precursor (450 ng; 10% of the amount added to the mitochondria) was digested under the same conditions in the presence of 50 μg detergent-lysed mitochondria (**right panel**). The lane marked St. contained 10% of the amount of precursor added to the mitochondrial samples. SDS-gel electrophoresis and fluorography were carried out as described (Eilers and Schatz, 1986).

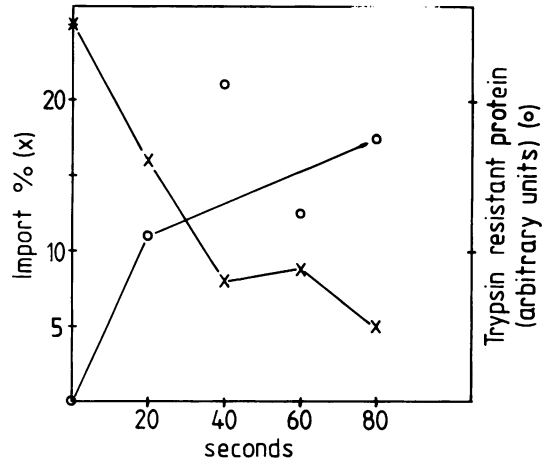

Fig. 7. Refolding of precursor abolishes its capacity for methotrexate-insensitive import. Native precursor (2.4×10^4 c.p.m.) was incubated in import buffer containing 5 M urea for 1 h at room temperature (Endo and Schatz, 1988); it was then diluted 10-fold into import buffer at 30°C containing 150 nM methotrexate. Simultaneously, or after the indicated times, mitochondria (200 μg protein) were added; after further incubation for 60 s, import was stopped by 10 μg/ml valinomycin. In a parallel experiment, precursor was diluted as above in methotrexate-containing import buffer and trypsin (83 μg/ml final concentration) was added simultaneously, or after the indicated times. After incubation for 1 min on ice, digestion was stopped by 1 mM PMSF. Mitochondria were re-isolated and analyzed by SDS−gel electrophoresis, fluorography and scanning. The figure shows the amount of mature form generated during the import reaction as % of added precursor and the amounts of trypsin-resistant DHFR-fragment in arbitrary units.

chondrial surface decreased 5-fold and subsequent import of the bound precursor decreased 3-fold, resulting in a 15-fold overall decrease (Figure 4). However, once the intermediate had been generated by energized mitochondria, its chase into an internalized, cleaved form was essentially potential-independent. This indicated that the intermediate had already passed the potential-sensitive step (Schleyer and Neupert, 1985). Finally, the bound intermediate could be imported at much lower temperatures than the native precursor; if the intermediate had been accumulated at 30°C

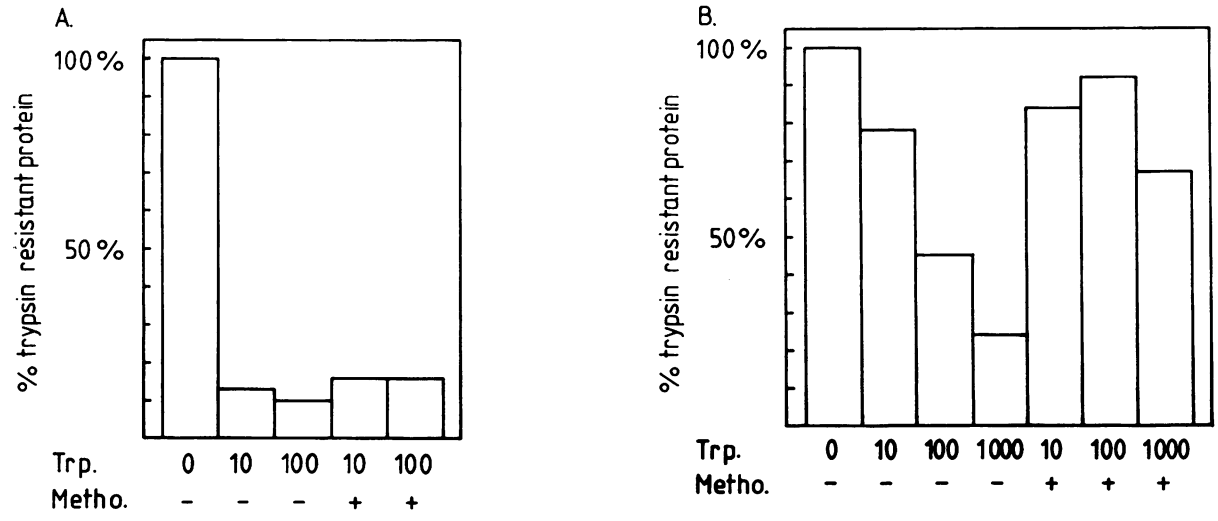

Fig. 8. The surface-bound intermediate refolds upon import. Mitochondria were incubated with native precursor under ATP-depleted conditions to accumulate the intermediate (see Figure 3). They were re-isolated, and half the mitochondria (containing the intermediate, **panel A**) were lysed in ice-cold 10 mM Hepes–KOH, 50 mM KCl and 0.5% octyl-POE. The lysate was either left on ice or incubated with the indicated amounts of trypsin (Trp.; μg/ml) in the absence or presence of 150 μM methotrexate (Metho.), respectively. After 10 min on ice, the digestion was stopped by the addition of 1 mM PMSF. The remaining half of the mitochondria were re-incubated in fresh import buffer containing an ATP regenerating system (see legend to Figure 3) and 10 μg/ml valinomycin for 5 min at 30°C. These mitochondria (**panel B**) were re-isolated and analyzed as in **panel A**. The graph shows the added amounts of precursor and the mature and trypsin-resistant forms generated. The amounts recovered without trypsin-digestion are arbitrarily set to 100%.

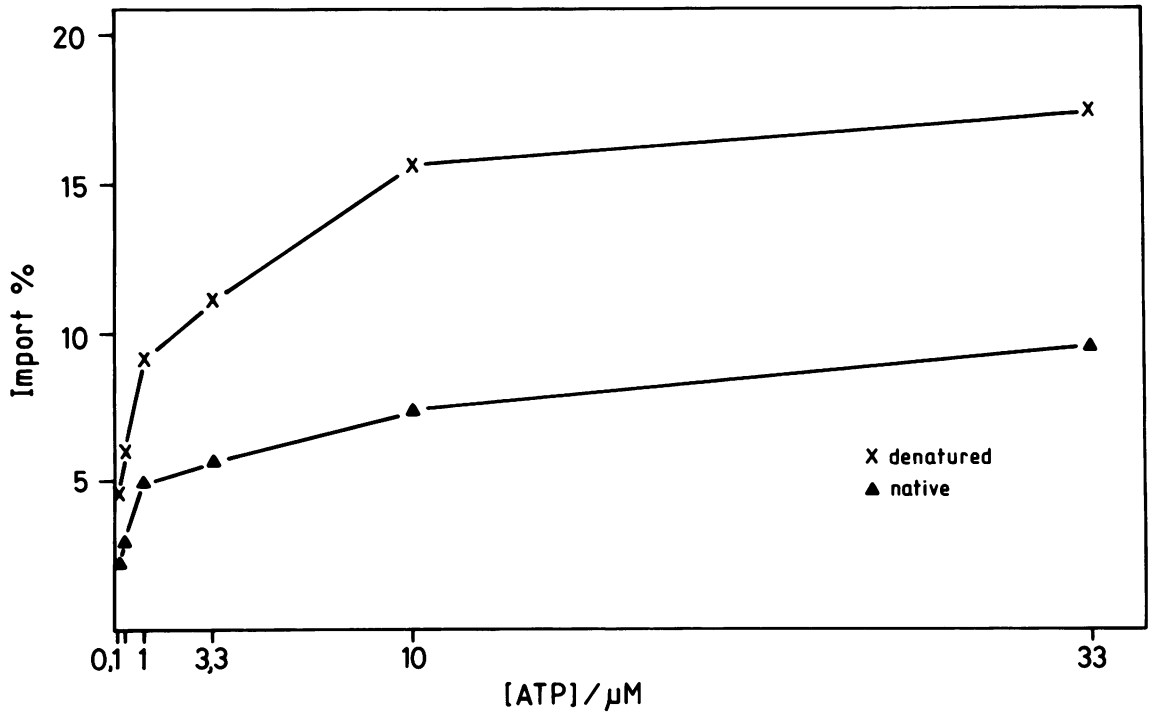

Fig. 9. Urea-denatured and native precursor require similar amounts of ATP for their import. Mitochondria (50 μg protein) were depleted of ATP (Eilers *et al.*, 1987) and incubated either with native or with urea-denatured precursor (450 ng; 7×10^7 c.p.m./mg) at 30°C in the presence of 25 mM sodium succinate, pH 7.0, 25 mM sodium malate, pH 7.0, 2.5 μg/ml efrapeptin and ATP-regenerating system containing 9 mM creatine phosphate, 2.8 U creatine kinase and the indicated concentrations of ATP. After 30 s (denatured precursor) or 10 min (native precursor), import was stopped by the addition of valinomycin to 5 μg/ml. Mitochondria were treated with proteinase K, re-isolated and analyzed by SDS gel electrophoresis and fluorography as described (Eilers and Schatz, 1986). The amount of protease-protected, mature form was determined by scanning.

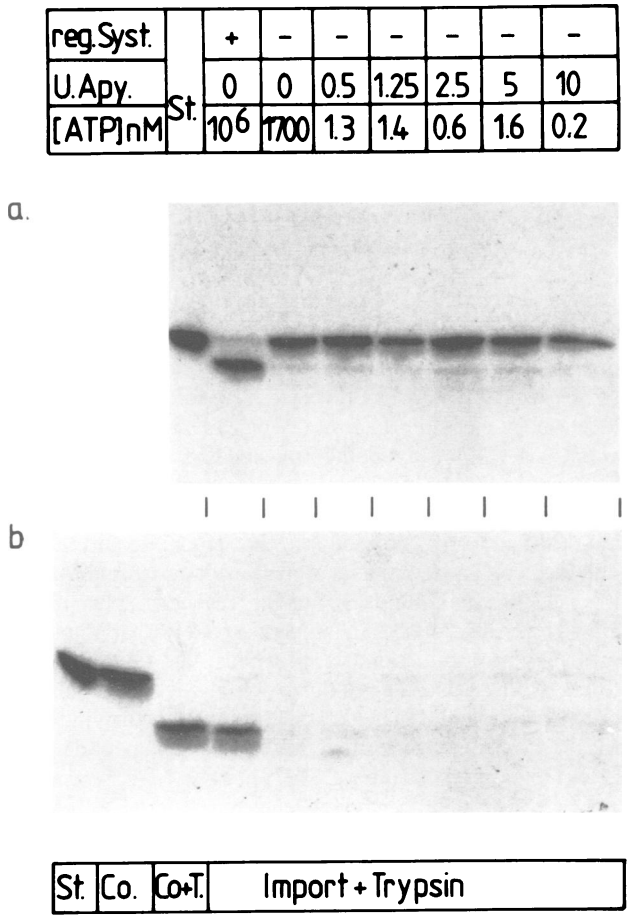

Fig. 10. The unfolded intermediate is formed in the absence of ATP. The surface-bound intermediate was generated from native precursor at 30°C as described in Figure 5 except that the incubation contained the indicated amounts of apyrase (Sigma; grade VIII) to deplete ATP (Pfanner and Neupert, 1986); after incubation for 10 min at 30°C, mitochondria were re-isolated. Half of each incubation was directly processed for gel electrophoresis (**panel a**). The remaining half was lysed in 50 mM KCl, 10 mM Hepes–KOH, pH 7.4, 0.5% octyl-POE and digested with 10 μg/ml trypsin for 10 min on ice; protease digestion was stopped by adding 1 mM PMSF and the samples were processed for electrophoresis (**panel b**). As a control, native precursor (10% of the amount added to mitochondria) was digested with the same amount of protease in the presence of lysed mitochondria (undigested, lane 'Co.'; digested, lane 'Co. + T.'). Appropriate aliquots of the supernatant of each import reaction were heated for 5 min to 95°C, immediately frozen in liquid nitrogen, and stored at −25°C. The ATP concentration of these samples was determined by a commercially available luciferase assay (Lumit-PM; Lumac bv; The Netherlands). The ATP samples used for the standard curve were treated identically to the actual import samples. The results are the average of at last three determinations.

in the absence of ATP, it could be chased into mitochondria in the presence of methotrexate even at 10°C (Figure 5). However, when ATP-depleted mitochondria were pre-incubated with the native precursor at 10°C, no unfolded intermediate was generated (Figure 5), even though the mitochondrial membrane potential (as measured with a potential-sensitive fluorophore, Sims *et al.*, 1974) was of similar magnitude at 10°C and at 30°C (not shown).

The unfolded state of the bound intermediate was also documented by the observation that the intermediate lacked the trypsin-resistance which is characteristic of native DHFR or the DHFR moiety of the native precursor (Figure 6). Mitochondria were allowed to accumulate the bound inter-

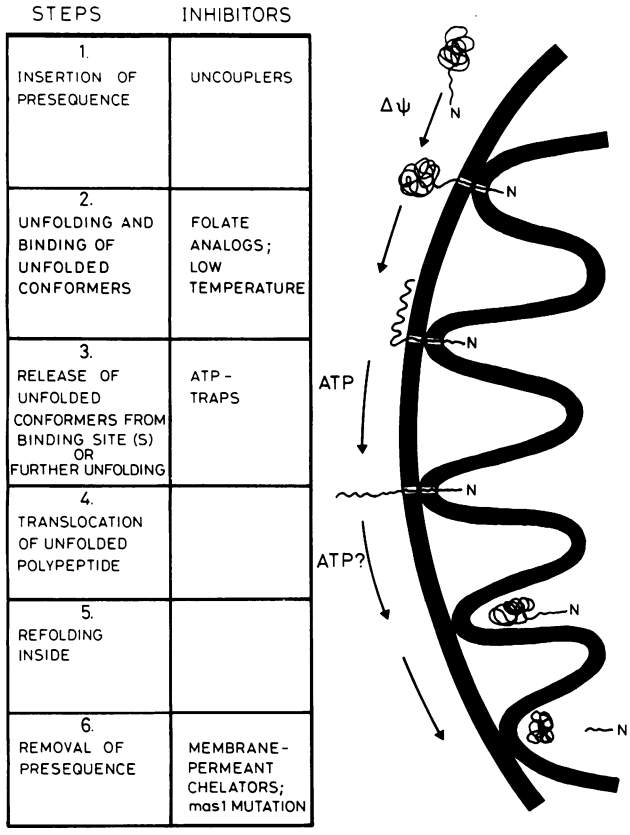

Fig. 11. A model for mitochondrial protein import. For description and references, see text. Import is assumed to occur at contact sites between the inner and outer mitochondrial membrane (Kellems *et al.*, 1975; Schleyer and Neupert, 1985).

mediate, re-isolated and lysed with the non-ionic detergent, octyl-POE. The lysate was incubated with increasing amounts of trypsin (left panel); as a control, native precursor was exposed to trypsin in the presence of lysed mitochondria under the same conditions (right panel). As reported before, the native precursor yielded a trypsin-resistant degradation fragment which appears to represent the DHFR moiety of the precursor (Eilers and Schatz, 1986). In contrast, the bound intermediate was completely digested even at low trypsin concentrations without appearance of a trypsin-resistant fragment.

In summary, import of the bound intermediate strikingly resembles import of the urea-denatured precursor with respect to resistance to methotrexate and low temperature, independence of a membrane potential, and dependence upon ATP. This indicates strongly that the native precursor is being unfolded at the mitochondrial surface before its transport across the mitochondrial membranes.

Refolding of urea-denatured precursor in the absence of mitochondria is accompanied by loss of methotrexate-resistant import

The precursor protein is maximally unfolded upon exposure to 5 M urea for 1 h at 23°C and refolds almost completely within a few minutes upon lowering the urea concentration to 0.5 M (Endo and Schatz, 1988). After this relatively gentle denaturation, rapid refolding of the precursor (measured here by the re-emergence of a trypsin-resistant DHFR moiety) was accompanied by the rapid loss of methotrexate-

resistant import into isolated mitochondria (Figure 7). Fast, methotrexate-resistant import is thus limited to incompletely folded forms of the precursor protein.

Import of the surface-bound intermediate is accompanied by refolding

When the surface-bound intermediate was chased into mitochondria by addition of ATP and uncoupler, it regained the trypsin-resistance typical of the native precursor. Addition of methotrexate increased trypsin-resistance still further, indicating that the imported precursor could bind this substrate analog (Figure 8). This refolding did not require removal of the presequence by the matrix protease (Böhni *et al.*, 1980, 1983; McAda and Douglas, 1982); we also observed it (not shown) with mitochondria isolated from the *mas 1* mutant which is deficient in this protease activity (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985).

Role of ATP

Work from several laboratories has shown that both ATP and an energized inner membrane are necessary for transport of proteins into mitochondria (see above). Different types of precursor chains, however, need different amounts of ATP (Pfanner *et al.*, 1987); in particular, the import of nascent, incompletely folded polypeptide chains still attached to tRNA does not require ATP (Verner and Schatz, 1987). Surprisingly, import of the urea-denatured precursor required about the same concentrations of ATP as import of the native precursor (Figure 9). In contrast, generation of the bound, unfolded intermediate from the native precursor was ATP-independent (Figure 10). Under the conditions of this experiment, the level of ATP in the supernatant at the end of the incubation was below 1 nM whereas that of precursor was 60 nM.

Discussion

We have provided evidence that the native precursor protein studied here is unfolded on the mitochondrial surface before it is translocated and that it refolds during or after translocation. A model consistent with our results is presented in Figure 11. We propose that import starts with an interaction of the presequence with the mitochondrial membranes, accompanied or followed by binding and stabilization of unfolded conformers of the precursor at sites on the mitochondrial surface. This initial step requires a potential across the inner membrane, is blocked by folate analogs or low temperature, does not require ATP, and appears to be a major rate-limiting step in the import of the native precursor. It could well depend on local movements within the mature part of the precursor protein. Steps 1 and 2 can be at least partly duplicated by exposing the folded precursor to cardiolipin-rich lipid vesicles (Endo and Schatz, 1988). Subsequent release of the bound intermediate into the import pathway requires ATP, is potential-independent, involves the unfolded precursor chain, and is followed by refolding of the protein and cleavage of the presequence inside the mitochondria. According to this model, denaturation of the precursor with urea eliminates step 2 as a rate-limiting step; import of nascent precursor chains (Verner and Schatz, 1987) bypasses steps 2 and 3, and import into mitochondria isolated from the protease-deficient *mas 1* mutant largely stops with step 5.

The role of ATP suggested here resembles the proposed role of ATP in the release of unfolded protein substrates from heat-shock proteins (Pelham, 1986). Indeed, import of proteins into mitochondria may well be mediated by heat-shock-like proteins. Alternatively, ATP might be required to complete the unfolding of the surface-bound intermediate, thereby allowing it to enter the import pathway. The precise role of ATP remains to be defined.

It is not clear to what extent unfolding of precursors takes place *in vivo*; with respect to temperature-dependence and speed, import of the urea-denatured precursor or of the surface-bound intermediate resemble the *in vivo* situation more closely than import of the native precursor. *In vivo*, some precursors may be prevented from folding before translocation by cytosolic factors (Wiech *et al.*, 1987; Pfanner *et al.*, 1987), or by being translocated cotranslationally (Kellems *et al.*, 1975). However, we show here that a membranous organelle can unfold at its surface precursors which are destined to be imported into that organelle and that the unfolded conformer exhibits properties of a true import intermediate. We find it unlikely that this reaction is physiologically irrelevant. Our data indicate strongly that import of proteins into mitochondria proceeds via unfolded conformers. They also suggest that refolding of the protein after translocation might provide at least part of the driving force for the transmembrane movement of the polypeptide chain (von Heijne and Blomberg, 1979).

Materials and methods

Mitochondrial import experiments

Mitochondria were prepared (Daum *et al.*, 1982) from the wild-type *Saccharomyces cerevisiae* strain D 273-10B and tested for import of the purified pCoxIV-DHFR fusion protein (Gasser *et al.*, 1982; Hurt *et al.*, 1984) as described (Eilers and Schatz, 1986).

Miscellaneous

Published methods were used for SDS–12% PAGE and immune blotting (Hurt *et al.*, 1985), protein measurement (BCA-method described by Pierce Chemical Co., USA), ATP-depletion of mitochondria (Eilers *et al.*, 1987) and purification of the pCoxIV-DHFR fusion protein (Eilers and Schatz, 1986). Unless stated otherwise, the native fusion protein was denatured by urea as outlined by Endo and Schatz (1988). Octyl-POE (octylpolyoxyethylene) and efrapeptin were kindly donated by Dr Jürg Rosenbusch (Biocenter, Basel) and Dr Henry Lardy (University of Wisconsin, Madison), respectively. All other experimental details are described in the figure legends.

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