Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP

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We have studied the post-translational import of incomplete precursor chains into isolated yeast mitochondria. The precursor was a fusion protein containing a mitochondrial presequence attached to mouse dihydrofolate reductase. *In vitro*-synthesis of the precursor was interrupted by the elongation inhibitor cycloheximide and the arrested nascent chains cosedimenting with ribosomes were released by EDTA. These incomplete chains were efficiently imported by isolated yeast mitochondria; their import resembled that of the complete precursor in requiring an energized inner membrane and a mitochondrial presequence. It differed from that of the completed precursor in its resistance to methotrexate (which only binds to correctly folded dihydrofolate reductase) and its independence of added ATP. The incomplete chains were also more sensitive to proteinase K than the completed precursor. We conclude that the incomplete chains were incompletely folded and suggest that the lack of tight folding caused import into mitochondria to become independent of added ATP. This implies that ATP may participate, directly or indirectly, in the unfolding of the precursor for its transport into mitochondria.

Key words: ATP/ATP-dependent protein unfolding/mitochondrial import/protein translocation/protein unfolding

Introduction

Protein translocation across several membrane systems requires ATP. This was shown for the *Escherichia coli* plasma membrane (Chen and Tai, 1985; Geller *et al.*, 1986), the endoplasmic reticulum (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Mueckler and Lodish, 1986), chloroplasts (Grossman *et al.*, 1980; Flüge and Hinz, 1986) and mitochondria (Pfanner and Neupert, 1986; Eilers *et al.*, 1987). It is, thus, likely that protein translocation across all membrane systems is dependent on ATP. With mitochondria and the bacterial plasma membrane, a transmembrane potential is needed as well (Wickner and Lodish, 1985).

What is the function of ATP in protein translocation? Since non-cleavable ATP analogs are inactive, ATP must apparently transfer its terminal phosphoryl group (see refs above). This raised several, mutually compatible possibilities. First, ATP could phosphorylate one or more participant(s) in the translocation event. Second, it could serve as an energy-donor for a membrane-spanning 'polypeptide transporter'. Third, it could provide energy to unfold the polypeptide before or during the translocation step.

Here we report evidence that is compatible with the third possibility. We show that post-translational import of incompletely folded precursor chains into isolated yeast mitochondria requires only an energized inner membrane, but no added ATP. In con-

trast, import of the completely folded precursor requires both an energized inner membrane as well as added ATP.

Results

The experimental approach

Isolated, respiring yeast mitochondria were allowed to import two forms of a precursor protein: the completed protein; and incompletely folded, truncated chains of the protein.

The precursor protein was a fusion protein containing the first 22 residues of the yeast cytochrome oxidase subunit IV precursor (pCOXIV; an imported mitochondrial protein) fused to mouse dihydrofolate reductase (DHFR; a cytosolic enzyme). This fusion protein (pCOXIV-DHFR) behaves like an authentic mitochondrial precursor protein *in vitro* and *in vivo*: its import into mitochondria is rapid and efficient, dependent on a membrane potential and ATP, and accompanied by proteolytic removal of the attached presequence (Hurt *et al.*, 1984a,b). The protein can be synthesized in *E. coli* in the presence of $^{35}\text{SO}_4^{2-}$, purified to near homogeneity, and imported into isolated, energized mitochondria in the absence of additional cell fractions (Eilers and Schatz, 1986).

For studying import of complete precursor protein, we used either the purified protein, or its completed, full-length chains that had been synthesized in a nuclease-pretreated reticulocyte lysate.

To prepare incompletely folded chains of this protein, we interrupted its synthesis *in vitro* by adding the elongation inhibitor, cycloheximide.

Fig. 1. Time course of synthesis of the precursor fusion protein pCOXIV-DHFR in a reticulocyte lysate. The fusion protein was synthesized by coupled transcription/translation in a nuclease-pretreated reticulocyte lysate in the presence of $[^{35}\text{S}]$ methionine at 25°C. At the indicated times (min), aliquots of the translation mixture were dissociated in SDS and analyzed by SDS-15% polyacrylamide gel electrophoresis and fluorography. The position of the completed fusion protein is indicated by the arrow.

Fig. 2. Incomplete chains generated by cycloheximide, but not those generated by puromycin, are solubilized by EDTA and include molecules larger than the completed precursor protein. The pCOXIV-DHFR fusion protein was synthesized by coupled transcription/translation as in Figure 1 for 7 min at 25°C. Protein synthesis was then stopped by adding either cycloheximide (to 100 µg/ml) or puromycin (to 3 mM). Samples were then treated as described below and analyzed by SDS–15% polyacrylamide gel electrophoresis and fluorography. **Lanes:** 1, transcription/translation without plasmid carrying the pCOXIV-DHFR fusion gene; 2, after synthesis for 7 min; 3 and 8, after synthesis for 7 min, addition of inhibitor, and an additional incubation for 7 min; 4 and 9, pellet obtained upon centrifuging the inhibited samples for 30 min at 130 000 g in a Beckman Airfuge; 5 and 10, supernatants from the centrifugation; 6 and 11, pellet obtained upon treating the inhibited samples with 10 mM EDTA and centrifugation as described above; 7 and 12, supernatants obtained after centrifuging the inhibited, EDTA-treated samples. Arrow, completed precursor; asterisk: region of the bands migrating more slowly than the completed bands (lanes 2–4, 7).

If synthesis was allowed to proceed at 25°C, incomplete chains of the fusion protein became detectable at 4 min and reached a plateau after 8 min (Figure 1). Completed chains were detectable only at 6 min (Figure 1) and continued to increase without a significant plateau for as long as 30 min (not shown).

By interrupting synthesis at 7 min with cycloheximide, we obtained a mixture of completed and incomplete chains (Figure 2, lane 2); arrest by cycloheximide was essentially complete since further incubation in the presence of this inhibitor did not result in further incorporation of ³⁵S into protein (lane 3). All radiolabeled bands represented chains of the pCOXIV-DHFR fusion as omission of the plasmid-borne fusion gene from the synthesis mixture yielded a blank fluorogram (lane 1).

If synthesis of the fusion protein was stopped with cycloheximide after 7 min and the mixture was centrifuged, all of the arrested nascent chains sedimented with the ribosomes whereas the completed chains were soluble (lanes 4 and 5). However, if the ribosomes were dissociated by 10 mM EDTA before centrifugation, both completed and incomplete chains of the fusion protein were quantitatively recovered in the supernatant (lanes 6 and 7). This supernatant was used as a source of C-terminally truncated, incompletely folded precursor chains. C-terminal truncation was documented by the results of SDS–polyacrylamide gel electrophoresis (Figure 1); evidence for incomplete folding of these chains will be presented in a later section.

The supernatant shown in lane 7 also contained variable amounts of completed chains of the precursor protein. These served as internal controls during the mitochondrial import. In some of the experiments, these completed chains were removed and replaced by radiolabeled purified fusion protein. The reasons for this will be discussed in a later section.

Fig. 3. RNase eliminates the slowly migrating bands detected in cycloheximide-arrested translation products. The COXIV-DHFR fusion protein was synthesized in a nuclease-treated reticulocyte lysate for 7 min at 25°C. An equal volume of Arrest Buffer (see Materials and methods) was added to stop the reaction, an aliquot was treated with 100 µg/ml RNase A (pretreated at 100°C for 10 min) for 10 min at 0°C and analyzed by SDS–15% polyacrylamide gel electrophoresis and fluorography. Equivalent amounts of the original translation reaction are shown. **Lanes:** 1, translation products; 2, translation products treated with 100 µg/ml RNase A. Arrow indicates completed precursor protein.

The fluorograms shown in Figure 1 and Figure 2, lanes 2–4 reveal labeled bands migrating more slowly than the completed fusion protein. These higher mol. wt bands cofractionate with the incomplete chains, not with the completed protein (Figure 2, lanes 4–7). We suspected that these bands represented incomplete chains that were still attached to tRNA since inhibition of translation by cycloheximide yields ribosome-bound peptidyl-tRNA (Colombo *et al.*, 1966; Godchaux *et al.*, 1967). To verify this, we arrested protein synthesis with puromycin; this inhibitor, in contrast to cycloheximide, binds to the C-terminus of nascent polypeptide chains in place of the appropriate tRNA and causes release of the prematurely-terminated chains from the ribosomes as puromycyl-peptides. Since puromycin (mol wt. 471.5) is much smaller than tRNAs (mol wt. $\sim 3 \times 10^4$), it should not generate labeled bands which are measurably larger than the completed fusion protein. This was indeed observed (Figure 2, lane 8). Unexpectedly, however, the incomplete puromycyl-peptides remained insoluble even after ribosomes had been dissociated by EDTA (Figure 2, lanes 9–11). This suggested that the incomplete chains generated under our conditions were inherently insoluble, presumably because they denatured quickly. In addition, many of these puromycyl-peptides may have adhered to tube walls, resulting in a decreased recovery compared to incomplete chains generated by cycloheximide (compare lanes 3 and 8). Aggregation of incomplete chains was apparently prevented by attaching a large and highly charged tRNA, but not the much smaller, and weakly charged, puromycin molecule.

This view was further supported by the observation that incubation with RNase obliterated the bands migrating more slowly than the completed fusion protein (Figure 3). We conclude that these bands are peptidyl-tRNA molecules and suggest that most, if not all of the incomplete chains obtained upon inhibition with cycloheximide are kept soluble by a C-terminally-attached tRNA.

Fig. 4. Import of incomplete precursor chains into mitochondria: requirement for an energized inner membrane and a mitochondrial presequence. The COXIV-DHFR fusion protein (arrow; lanes 1–5) or authentic mouse DHFR (asterisk; lanes 6 and 7) were synthesized by coupled transcription/translation in a nuclease-treated reticulocyte lysate for 7 min at 25°C in the presence of [³⁵S]methionine. Protein synthesis was stopped by adding an equal volume of Arrest Buffer (Materials and methods). Nascent chains were released from ribosomes by 10 mM EDTA, and 30 µl aliquots of the arrested mixture (containing both completed and incomplete precursor chains) were incubated with 200 µg of isolated yeast mitochondria for 20 min at 25°C (see Materials and methods). Where indicated, mitochondria were de-energized by 2 µg/ml valinomycin. The mitochondria were then re-isolated by centrifugation and analyzed by SDS–15% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were first treated with 250 µg/ml proteinase K for 15 min at 0°C and then with 1 mM phenylmethylsulfonyl fluoride before electrophoretic analysis. Lanes: 1, 35% of the labeled pCOXIV-DHFR chains (both completed and incomplete) which were added to the mitochondrial samples shown in lanes 2–5; 2, after incubation with energized mitochondria; 3, energized mitochondria, treated with proteinase K; 4, after incubation with de-energized mitochondria; 5, de-energized mitochondria, treated with proteinase K; 6, 35% of the labeled DHFR chains (both completed and incomplete) which were added to the mitochondrial sample shown in lane 7; 7, after incubation with energized mitochondria. The horizontal bars between lanes 5 and 6 denote the position of the following mol. wt markers (listed from top to bottom): ovalbumin (46 kd); carbonic anhydrase (30 kd); lactoglobulin A (18.37 kd); cytochrome c (12.3 kd).

Import of incomplete precursor chains into mitochondria

A cycloheximide-arrested reticulocyte lysate containing both incomplete and complete precursor chains was treated with EDTA to release incomplete chains from ribosomes and incubated with isolated yeast mitochondria. Figure 4 shows that, in the presence of a membrane potential, both the completed and the incomplete precursor chains were imported into mitochondria as reflected by their resistance to externally-added proteinase K (lanes 2 and 3). The incomplete chains were imported into mitochondria even more efficiently than the completed chains. Import of the completed precursor was clearly accompanied by cleavage of the presequence by the matrix protease, resulting in a protease-protected 'mature' protein. We have not yet determined if a

Fig. 5. Isolation of incomplete precursor chains. The pCOXIV-DHFR fusion protein (arrow) was synthesized *in vitro* as described in Figure 2. An equal volume of Arrest Buffer (see Materials and methods) was added and the mixture was centrifuged for 30 min at 130 000 g at 4°C. The pellet (consisting mostly of ribosomes) was resuspended in 20 mM Hepes pH 7.4, 5 mM MgCl₂, 92 units/ml yeast hexokinase, 56 mM glucose and incubated for 15 min at 0°C (in order to remove any ATP). It was then centrifuged a second time as above; the pellet was resuspended in 20 mM Hepes pH 7.4, 10 mM EDTA and incubated for 10 min at 0°C to dissociate ribosomes. Residual particles were removed by a third centrifugation step as above and the final supernatant was used as a source of released incomplete precursor chains. Aliquots of each sample (corresponding to the same amount of initial translation mixture) were analyzed as in Figure 2. Lanes: 1, total translation mixture; 2, supernatant from first spin; 3, pellet from first spin; 4, supernatant from second spin (after ATP-depletion); 5, pellet from second spin; 6, supernatant from third spin (containing released incomplete chains); 7, pellet from third spin.

similar cleavage occurs with the nascent chains, but consider this likely.

The import of both completed and incomplete precursor chains had an absolute requirement for a membrane potential as neither were protected from externally-added protease if the mitochondria had been treated with the uncoupler valinomycin (lanes 4 and 5). Under these conditions, both types of chains bound to mitochondria, but were not imported. The mol. wt distribution of the incomplete chains bound to uncoupled mitochondria differed from that of chains imported into energized mitochondria. This difference need not reflect processing of imported incomplete chains; it may be caused by non-specific proteolytic degradation of chains bound to the mitochondrial surface.

In order to determine if import of the incomplete precursor chains into mitochondria was dependent on the presence of the subunit IV presequence, authentic mouse DHFR was translated *in vitro* (lane 6). The translation products containing both the completed DHFR protein and its incomplete chains were then incubated with energized mitochondria. Neither completed DHFR nor its incomplete chains interacted with energized mitochondria (Figure 4, lane 7). Thus, import of incomplete chains of the pCOXIV-DHFR fusion protein into isolated mitochondria is both potential-dependent and presequence-dependent.

Isolation of ATP-depleted incomplete precursor chains

In order to check if ATP was required for import of incomplete precursor chains, we isolated these incomplete chains free of ATP. We exploited the observation that cycloheximide-arrested

Fig. 6. Only the completed, but not the incomplete precursor chains require added ATP for import into isolated yeast mitochondria. Radiolabeled incomplete pCOXIV-DHFR chains (24 μ l; isolated as in Figure 5) were mixed with radiolabeled purified completed pCOXIV-DHFR (8 μ l). The mixture was added to 225 μ g of yeast mitochondria in Succinate Import Buffer (see Materials and methods) which was supplemented with 12 mM glycerol and 23 μ g/ml efrapentin. Where indicated, 2 mM ATP, 2 units/ml of glycerol kinase or 2 μ g/ml valinomycin (to uncouple mitochondria) were also present. Two samples were incubated without mitochondria to check for aggregation of the labeled precursor chains. All samples (total volume 200 μ l) were incubated in air as shallow layers in plastic tubes for 20 min at 25°C and centrifuged for 10 min at 15 000 r.p.m. in an Eppendorf centrifuge; the pellets were analyzed by fluorography. Where indicated, mitochondria were treated with proteinase K (with or without 0.5% Triton X-100) followed by phenylmethylsulfonyl fluoride (as outlined in Figure 4) before electrophoretic analysis. **Lanes:** 1, 20% of the mixture of completed and incomplete precursor chains that was added to the samples shown in lanes 2–11; 2, after incubation with respiring mitochondria, plus ATP; 3, respiring mitochondria, ATP, proteinase K; 4, respiring mitochondria, ATP, proteinase K, Triton; 5, same as 2, but no mitochondria added; 6, respiring mitochondria plus glycerol kinase; 7, respiring mitochondria, glycerol kinase, proteinase K; 8, respiring mitochondria, glycerol kinase, proteinase K, Triton; 9, same as 6, but no mitochondria added; 10, uncoupled mitochondria, ATP, proteinase K; 11, uncoupled mitochondria, glycerol kinase, proteinase K. Arrow indicates purified precursor protein. The band below the completed precursor in lane 1 is a contaminant present in some precursor preparations and does not appear to interact with mitochondria.

incomplete chains remained associated with ribosomes and could be separated from other components of the translation reaction by sedimentation (see Figure 2). A nuclease-pretreated reticulocyte lysate was allowed to synthesize pCOXIV-DHFR for 7 min; cycloheximide was then added and the mixture was centrifuged to pellet ribosomes, together with their attached incomplete precursor chains. The supernatant fraction (which contained the completed, released precursor) was removed; the ribosomal pellet was resuspended in buffer containing MgCl_2 , treated with hexokinase and glucose to hydrolyze any residual ATP, and recentrifuged to sediment ribosomes. As seen in Figure 5, the incomplete precursor chains were quantitatively sedimented with ribosomes before (lane 3) and after (lane 5) the ATP depletion step. In order to solubilize the incomplete precursor chains from the ribosomes, the ATP-depleted ribosomal pellet was resuspended in buffer containing 10 mM EDTA. Following a final centrifugation, the incomplete chains were found to be in the supernatant fraction (lane 6).

The incomplete chains isolated by this procedure still had the electrophoretic mobility shown in Figures 1 and 2, in that some of them migrated more slowly than the completed precursor. Therefore, the steps involved in the isolation of ATP-depleted

Fig. 7. Preincubation of mitochondria with completed precursor does not abolish subsequent import of incomplete precursor chains in the absence of added ATP. Purified pCOXIV-DHFR fusion protein and its isolated incomplete chains were added to respiring yeast mitochondria as in Figure 6 except that the order of additions and the incubation times were varied as indicated below. All mitochondrial samples were treated with proteinase K before being analyzed as in Figure 6. **Lanes:** 1, incomplete chains mixed with completed precursor (20% of the amounts added to the samples shown in lanes 2–6); 2, plus ATP; incomplete and completed chains added together and incubated with mitochondria for 30 min at 25°C; 3, no added ATP, plus glycerol kinase; incomplete and completed chains added simultaneously and incubated with mitochondria for 30 min; 4, plus ATP; only completed chains added for 10 min, followed by addition of incomplete chains and an additional incubation for 20 min; 5, plus ATP; completed chains added for 10 min, followed by addition of incomplete chains and an additional incubation for 20 min. Arrow indicates purified precursor protein.

incomplete chains described here did not appear to hydrolyze the ester bond of the peptidyl-tRNA molecules.

The ATP-depleted incomplete precursor chains were then mixed with the ^{35}S -labeled completed precursor protein which had been purified from *E. coli* transformants. This artificially-generated mixture of purified precursor and its ATP-depleted incomplete chains offered distinct advantages over removing ATP directly from a reticulocyte lysate containing both types of chains. First, isolation of incomplete chains by our method involved removal of the ribosome-associated chains from the lysate followed by two washes with buffer. As the ATP-trapping system was only present in the first washing solution, this system, together with most soluble components of the reticulocyte lysate, were removed before the import assay. Thus, import could be measured by adding to mitochondria only the purified precursor and its incomplete chains, as well as ribosomal components released by EDTA. Another advantage of using a mixture of purified precursor and its isolated incomplete chains was that each of them could be added to the import reaction separately.

Incomplete precursor chains are imported without added ATP

Isolated yeast mitochondria were suspended in Succinate Import Buffer (see Materials and methods); the respiration substrate succinate allows mitochondria to maintain an electrochemical potential without added ATP. In addition, efrapentin was included to block respiration-driven ATP synthesis. A mixture of purified precursor protein and its *in vitro*-synthesized incomplete chains was then added either together with ATP or with an ATP-trapping system consisting of glycerol kinase and glycerol. In the presence

Fig. 8. The addition of ATP does not affect the rate or the extent of import of incomplete precursor chains. Purified pCOXIV-DHFR fusion protein and its isolated incomplete chains were added to respiring yeast mitochondria as in Figure 6 except that the incubation times were varied as indicated. The import reactions were terminated by the addition of 2 μ g/ml valinomycin and chilled to 0°C. All samples were treated with proteinase K before being analyzed as in Figure 6. Import of incomplete precursor chains was determined by densitometry and is presented in arbitrary units. In this experiment, import of the purified completed precursor was stimulated 6.4-fold by the addition of ATP at the final time point. Open triangles, import of incomplete precursor chains without the addition of ATP; closed circles, import of incomplete precursor chains with the addition of ATP.

of added ATP, both the completed precursor and its incomplete chains were imported into mitochondria and became inaccessible to externally-added protease (Figure 6, lane 3). This protease-resistance was abolished when the mitochondrial membranes were disrupted with Triton X-100. In that case, the imported DHFR moiety was converted to a distinctly smaller proteolytic fragment that is quite resistant to further degradation (cf. also van Loon *et al.*, 1986). In the absence of added ATP, however, import of the completed precursor was virtually abolished whereas that of the incomplete precursor chains was only slightly diminished (compare lanes 3 and 7).

When mitochondria were uncoupled by valinomycin/ K^+ , import of both completed and incomplete precursor chains was prevented regardless of whether or not ATP had been added (lanes 10 and 11). Therefore, the import of completed precursor required both ATP and an energized membrane whereas the import of incomplete precursor chains required only the membrane potential.

We considered the possibility that despite steps to exclude ATP from the import assay, a small pool of ATP may have remained. If incomplete chains were imported into mitochondria more rapidly than the completed precursor they might well require ATP for import, but deplete such a small ATP pool before the completed precursor had begun to move into mitochondria. To rule out this possibility, mitochondria were first allowed to import the completed precursor in the absence of added ATP for 10 min; only then were they presented with the incomplete chains. In this experiment, any residual traces of ATP should have been consumed for import of the completed precursor before the incomplete chains had been introduced into the reaction. The results of this experiment are presented in Figure 7; lanes 2 and 3 show import in the presence and absence of added ATP when completed precursor and incomplete chains were added together. Lane 4 shows the import of completed precursor alone for 10 min in the presence of ATP, indicating that the incubation time had been

Fig. 9. Incomplete precursor chains are more sensitive to digestion by proteinase K than the completed chains. A mixture of radiolabeled purified pCOXIV-DHFR precursor and its isolated incomplete chains was incubated for 20 min at 0°C with the indicated final concentrations of proteinase K (μ g/ml) in 20 mM Hepes pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT. Digestion was stopped by adding phenylmethylsulfonyl fluoride to 1 mM and the digestion products were analyzed by SDS-15% polyacrylamide gel electrophoresis and fluorography. Arrow indicates purified precursor protein.

sufficient to allow substantial import of completed chains before addition of the incomplete chains. Finally, when incomplete chains were added 10 min *after* the completed precursor in the presence (lane 5) or absence (lane 6) of ATP, the result was the same as before: import of the completed chains was dependent on ATP, whereas that of the incomplete chains was independent of added ATP (compare lanes 3 and 6). Therefore, the different ATP requirements of the two types of chains cannot be attributed to a kinetic advantage of incomplete chains over their completed counterparts.

As the incomplete chains are imported much more rapidly than the completed ones, their apparent independence on added ATP could well be an artefact: their import could already have been maximal at the first time point of our measurements. The time-course experiment shown in Figure 8 excludes this possibility; it shows that neither the rate nor the final extent of import of the incomplete chains were significantly affected by the addition of ATP.

Incompletely synthesized precursor chains are incompletely folded
Mouse DHFR has a compact globular structure (Volz *et al.*, 1982) which is highly resistant to trypsin and other proteases (Eilers and Schatz, 1986). To a large measure, this is also true of the pCOXIV-DHFR fusion protein: its DHFR moiety is quite resistant to various proteases, catalyzes DHFR activity, and binds DHFR-specific ligands such as methotrexate (Eilers and Schatz, 1986). As a result, import of the fusion protein into isolated mitochondria is blocked by these ligands, apparently because they prevent the mandatory partial or complete unfolding of DHFR during its passage across the mitochondrial membranes.

It seemed reasonable to suspect that the DHFR portion of the incompletely synthesized fusion protein would not exhibit the stable tertiary conformation of the completed precursor. Such incomplete folding should manifest itself in two ways: the incomplete chains should be more sensitive to protease than the

Fig. 10. Import of the completed, but not the incomplete, precursor chains is inhibited by methotrexate. Radiolabeled incomplete pCOXIV-DHFR chains were mixed with radiolabeled purified pCOXIV-DHFR fusion protein as in Figure 6. The mixture was then incubated for 10 min at 25°C either in the presence or the absence of 250 mM methotrexate. Aliquots were added to 225 μ g of yeast mitochondria in Succinate Import Buffer supplemented with 2 mM ATP. Import of the radiolabeled precursor chains into mitochondria was allowed to proceed for 20 min as in Figure 6. The mitochondria were re-isolated by centrifugation and analyzed by SDS-15% polyacrylamide gel electrophoresis followed by fluorography. Where indicated, mitochondria were treated with proteinase K (with or without 0.5% Triton X-100) followed by 1 mM phenylmethylsulfonyl fluoride before electrophoretic analysis as in Figure 6. **Lanes:** 1, mitochondria incubated with untreated precursor mixture; 2, mitochondria, untreated precursor mixture, proteinase K; 3, mitochondria, untreated precursor mixture, proteinase K, Triton; 4-6, same as 1-3 except that the precursor mixture had been preincubated with methotrexate. The arrow indicates the purified, full-length precursor protein. The polypeptide generated by protease K in the presence of Triton is a proteolytic fragment of DHFR that is relatively resistant to further degradation (van Loon *et al.*, 1986).

completed ones, and their import into mitochondria should be resistant to methotrexate. Both predictions proved to be correct.

The increased protease-susceptibility of the incomplete chains is shown in Figure 9. A mixture of incomplete chains and purified, completed precursor (the same mixture which had been used in the import experiments of Figures 6 and 7) was divided into aliquots which were then incubated with increasing concentrations of proteinase K. After the protease had been inhibited with phenylmethylsulfonyl fluoride, each aliquot was analyzed for proteolytic degradation by SDS-polyacrylamide gel electrophoresis and fluorography. With the completed precursor, increasing amounts of protease yielded a stable product of lower mol. wt; this product was most likely the DHFR moiety from which the subunit IV presequence had been cleaved off; this proteolytic intermediate, like authentic mouse DHFR, resists further degradation even at the highest protease concentrations used in this experiment (van Loon *et al.*, 1986; see also Figure 6). In contrast, increasing protease levels degraded the incomplete chains without the appearance of any stable intermediate. At proteinase K concentrations of between 1 and 2 μ g/ml, the incomplete chains were digested to peptides which were no longer displayed by our electrophoretic system. This was also the protease level which removed the presequence from the completed precursor protein.

Fig. 11. Working model on the individual steps of post-translational protein transport into the mitochondrial matrix. The positively charged presequence is indicated by three + signs. Some steps (e.g. 3 and 4, or 5, 6 and 7) might occur simultaneously. Although not shown here, ATP-dependent unfolding may occur within the membrane-embedded translocation machinery. Loose folding of the newly-made mitochondrial precursor protein is suggested based on work by Chien and Freeman, 1986, membrane potential-dependent insertion of the presequence on the basis of work by Schleyer and Neupert (1986) and insertion of the presequence into the lipid bilayer on the basis of work reported by Roise *et al.* (1986) and of unpublished work. According to this model, the incompletely folded precursor chains studied here bypass step 5.

Import of the incomplete precursor chains into mitochondria is resistant to methotrexate

Import of DHFR-containing fusion proteins into mitochondria is blocked by antifolates such as methotrexate. These substrate analogs tightly bind to the catalytic site on DHFR, thereby stabilizing its three-dimensional structure. This suggests that translocation of DHFR across mitochondrial membranes requires at least partial unfolding of the protein (Eilers and Schatz, 1986).

If the incomplete pCOXIV-DHFR chains studied here are indeed incompletely (or incorrectly) folded, they should not exhibit the normal catalytic site of authentic DHFR and fail to bind methotrexate. As a result, their import into mitochondria should differ from that of the completed chains in being resistant to methotrexate. This proved to be correct (Figure 10): import of the incomplete chains (even the longest ones displayed by the gels) is only slightly affected whereas import of the completed precursor protein is strongly inhibited. This result strongly in-

icates that the incomplete precursor chains generated by cycloheximide inhibition have not attained the three-dimensional structure of enzymically active DHFR.

Discussion

Import of incomplete precursor chains into mitochondria

In this study we show that incompletely synthesized precursor polypeptides can be imported into isolated mitochondria. This supports earlier findings with intact yeast cells which had suggested that nascent, ribosome-bound precursor chains can enter mitochondria, at least under certain conditions (Kellems *et al.*, 1975; Ades and Butow, 1980; Reid and Schatz, 1982; Suissa and Schatz, 1982). In addition, the present data reaffirm the view that the information for transporting proteins into mitochondria is located in amino-proximal regions of the transported proteins (Douglas *et al.*, 1984; Hase *et al.*, 1984; Hurt *et al.*, 1984a,b).

Our results strongly imply that the incompletely-synthesized precursor chains are incompletely folded. While this appears obvious with very short chains it was less evident with those that had nearly attained their final size. For example, the protease-digestion patterns of Figure 9 showed that incomplete chains migrating only slightly faster than the completed precursor were as protease-sensitive as much shorter chains. In addition, Figure 10 shows that import of even the longest of the incomplete chains was not inhibited by methotrexate. This can not be explained by simply assuming that the residues of the methotrexate-binding site had not yet been synthesized, as the most C-terminal residue involved in ligand binding is located some 40 residues from the C-terminus (Howell *et al.*, 1986).

This could have been rationalized by the assumption that DHFR can only fold correctly after the extreme C-terminal region has been synthesized (cf. e.g. Taniuchi, 1970). However, we consider it much more likely that the incomplete chains did not fold correctly because their C-terminus was still linked to tRNA. This is strongly suggested by the known mode of action of cycloheximide, the RNase sensitivity of the bands larger than the completed precursor and the absence of these larger bands from incomplete chains produced by puromycin.

Since tRNA should not be able to pass membranes, the imported incomplete chains might have become 'stuck' across the mitochondrial membrane(s) with the peptidyl moiety inside and the tRNA moiety outside the membrane barrier(s). Alternatively, the peptidyl-tRNA linkage might be hydrolyzed by non-specific esterases associated with mitochondria.

In summary, the combined evidence strongly suggests that the incomplete precursor chains generated by cycloheximide-arrest are, in fact, peptidyl tRNA molecules.

Implications for the mechanism of protein translocation into mitochondria

Mitochondrial import of the incomplete chains, like that of the completed precursor, requires an energized inner membrane and a mitochondrial presequence. However, under conditions preventing oxidative phosphorylation, only the incomplete chains can be imported in the absence of added ATP. We cannot rigorously exclude that import of these chains is completely ATP-independent since even mitochondria preincubated with efrapetin (an inhibitor of mitochondrial ATP synthase) retain low levels of ATP (M.Eilers, unpublished). At the very least, however, import of the incomplete chains requires much less ATP than that of the completed ones.

This striking result is most easily explained by the assumption

that ATP is necessary to unfold precursor proteins before or during their import into mitochondria. The existence of ATP-dependent protein unfolding mechanisms has been proposed before (Rothman and Kornberg, 1986). It would be in harmony with recent evidence that protein translocation across membranes always requires ATP (Zimmermann and Meyer, 1986) and, in some instances, is correlated with or dependent upon at least partial unfolding of the transported protein (Randall and Hardy, 1986; Eilers and Schatz, 1986). It also fits the observation that, with mitochondria, ATP supports protein translocation at a site outside the inner membrane (Eilers *et al.*, 1987).

Although the longest incomplete precursor chains were imported into isolated mitochondria without the addition of ATP, it was sometimes observed that the shorter chains were imported more efficiently under these conditions (see Figure 7). It is therefore possible that although the longest incomplete chains cannot obtain the stable, enzymatically active conformation of the completed precursor, they may assume conformations of greater stability than the shorter chains.

Figure 11 summarizes our current working hypothesis on how a precursor protein is transported post-translationally from the cytosol into the mitochondrial matrix. According to this model, the energy for unidirectional transport of the polypeptide chain is provided by ATP which unfolds the protein on the cytosolic-side of the membrane, generating an energy-rich, unstable conformation. Exergonic tight folding of the polypeptide chain on the opposite side of the membrane(s) would drive transport to completion. We suggest that ATP-dependent unfolding might also be involved in protein translocation across other membrane systems.

Materials and methods

Protein import into isolated mitochondria

Isolated yeast mitochondria were incubated with radiolabeled proteins as described by Gasser *et al.* (1982). The incubation (total volume 0.2 ml) contained 200 µg mitochondrial protein, 30 µl cycloheximide-arrested reticulocyte lysate containing radiolabeled translation products, 40 mM KCl and, where indicated, 2 µg/ml valinomycin. After 20 min at 25°C, the mitochondria were re-isolated by centrifugation through a sucrose cushion. For digestion of externally-located polypeptides following the import reaction, the mitochondria were incubated for 15 min at 0°C with 250 µg/ml proteinase K. The protease was inhibited with 1 mM phenylmethylsulfonyl fluoride and the mitochondria were re-isolated as above.

In experiments where the effect of ATP on import was analyzed and in the experiment shown in Figure 10, 225 µg mitochondria (200 µl total volume) were incubated in a shaking waterbath for 20 min at 25°C as a shallow layer in open plastic tubes in Succinate Import Buffer (0.6 M sorbitol, 40 mM KCl, 50 mM sodium succinate, 0.5 mM dithiothreitol, 10 mM MgCl₂, 1 mM methionine, 20 mM Hepes pH 7.4). In addition, 23 µg/ml efrapetin was included in each reaction. The import reactions contained either 2 mM ATP with 2.5 mM phosphoenol pyruvate and 5 units/ml pyruvate kinase or 2 units/ml of glycerol kinase to trap any ATP present. Re-isolation of mitochondria, proteinase K treatment, and valinomycin treatment were as above.

In vitro transcription/translation

A plasmid encoding the first 22 residues of cytochrome oxidase subunit IV fused to mouse cytosolic dihydrofolate reductase (Hurt *et al.*, 1984b) was transcribed *in vivo* according to Wiedmann *et al.* (1984). Transcripts were then translated in a nuclease-treated reticulocyte lysate supplemented with hemin, creatine phosphate, creatine kinase, the 19 amino acids except methionine, 130 mM KCl, 2 mM MgCl₂ (Pelham and Jackson, 1976) as well as 250 µg/ml calf-liver tRNA, 50 units of human placental RNase inhibitor/ml and 1 mCi of [³⁵S]methionine (>1000 Ci/mmol) per ml (Hurt *et al.*, 1984a). Translation was performed at 25°C, usually for 7 min, at which time an equal volume of Arrest Buffer (200 µg/ml cycloheximide, 2 mM MgCl₂, 20 mM Hepes pH 7.4) was added. Alternatively, after 7 min incubation, the reaction was made 3 mM in puromycin and incubated again for several minutes. To release incomplete chains from ribosomes, 10 mM EDTA was added to the cycloheximide-arrested reactions. The translation products were kept on ice for use in import reactions.

Other methods

Published procedures were used to isolate mitochondria from the wild-type *Saccharomyces cerevisiae* strain D273-10B (Daum *et al.*, 1982), to purify ³⁵S-labeled pCOXIV-DHFR fusion protein (Eilers and Schatz, 1986), to prepare a nuclease-pretreated reticulocyte lysate (Pelham and Jackson, 1976), to analyze labeled translation products by SDS–15% polyacrylamide gel electrophoresis and fluorography (Hurt *et al.*, 1984a). Fluorographs were scanned using a Camag TLC Scanner II and a Camag SP4290 integrator. Protein was measured by the BCA procedure as described in a company brochure ('Previews') of Pierce Chemical Co.

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