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A Matrix ATP Requirement for Presequence Translocation across the Inner Membrane of Mitochondria*

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Douglas M. Cyr‡, Rosemary A. Stuart§, and Walter Neupert¶

From the Institut für Physiologische Chemie der Universität München, Goethestrasse 33, D-80336 München, Germany

The mitochondrial presequence initiates protein translocation across the inner membrane of mitochondria in a $\Delta\psi$-dependent step. We have investigated the role of matrix ATP in this process. When matrix ATP was reduced to interfere with the function of mitochondrial heat shock protein 70, presequence translocation across the inner membrane was strongly inhibited. This was accompanied by the accumulation of an import intermediate that was unprocessed and accessible to protease added to the intact mitochondria. Both $\Delta\psi$ and matrix ATP were required for further translocation of this intermediate into the matrix. When ATP levels are insufficient to support protein import, it appears that the presequence becomes translocated across the inner membrane, but $\Delta\psi$ does not maintain it in the matrix. Presequence translocation across the inner membrane is thus a reversible reaction, and a step dependent on matrix ATP is required to make it unidirectional. Based on these observations, a model on the role of $\Delta\psi$, mthsp70, and matrix ATP in presequence translocation across the inner membrane is presented.

Most mitochondrial proteins are synthesized as precursors with an amino-terminal presequence that is cleaved upon import (Hartl *et al.*, 1989). The presequence appears to play multiple roles in mitochondrial protein import. First, it targets precursor proteins to the mitochondrial import receptor complex of the outer membrane (Söllner *et al.*, 1989, 1990; Hines *et al.*, 1990; Kiebler *et al.*, 1990). Second, after transfer through the outer membrane, the presequence is thought to be recognized by an independent inner membrane import apparatus (Hwang *et al.*, 1989; Glick *et al.*, 1991; Pfanner *et al.*, 1992). Finally, membrane potential-dependent ($\Delta\psi$) transfer of the presequence initiates precursor translocation across the inner membrane (Schleyer *et al.*, 1985).

The mechanism of protein translocation across the inner membrane is poorly understood. A component of the putative inner membrane translocation apparatus through which the presequence could pass en route to the matrix has been identified (Maarse *et al.*, 1992; Scherer *et al.*, 1992). Protein translocation into the matrix is facilitated by ATP-dependent interaction of the precursor protein with mitochondrial hsp70[1] (mthsp70) (Kang *et al.*, 1990; Scherer *et al.*, 1990). It is, however, not clear whether the initial translocation of the presequence is driven in a unidirectional fashion by $\Delta\psi$ or whether mthsp70 also assists in this process.

To examine the potential role of mthsp70 in presequence translocation across the inner membrane, an import intermediate that accumulates with matrix ATP-depleted mitochondria was characterized. The intermediate was unprocessed and accessible to protease added to the intact mitochondria. In order to become further translocated into mitochondria, both $\Delta\psi$ and matrix ATP were required. Thus, matrix ATP appears to be required in conjunction with $\Delta\psi$ for efficient presequence translocation across the inner membrane.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria—Wild type mitochondria were isolated by established procedures from *Saccharomyces cerevisiae* strain D273-10B, which was grown in a semi-synthetic lactate media (Daum *et al.*, 1982). "ΔATP1 mitochondria" were isolated from *S. cerevisiae* strain W303aΔATP1 grown on YPGalactose media. The ATP1 gene, which encodes the α subunit of the F_1-ATPase, is disrupted in W303aΔATP1. The mitochondria isolated from this strain do not have a functional F_1-ATPase, cannot regenerate matrix ATP, and therefore have naturally low matrix ATP levels.

Protein Import into Matrix ATP-depleted Mitochondria—To reduce matrix ATP levels in wild type mitochondria, the following protocol was used.[2] Briefly, mitochondria (40 μg) were incubated in 200 μl of import buffer (500 mM sorbitol, 50 mM Hepes, pH 7.2, 80 mM KCl, 10 mM MgOAc, 2 mM potassium phosphate, and 3% bovine serum albumin) for 3 min at 25 °C to promote turnover of preexisting matrix ATP. Then carboxyatractyloside (5 μM), oligomycin (20 μM), NADH (2 mM), and ATP (2 mM) were added in successive order, with each addition being followed by a 3-min incubation period at 25 °C. Import reactions were then started by addition of ^{35}S-labeled precursor proteins in reticulocyte (final concentration of 1.5–3.0% lysate), incubated for 5 min at 25 °C, and stopped by transfer to ice. Samples treated as above are termed "– matrix ATP". Samples termed "+ matrix ATP" were treated identically except that oligomycin was omitted. Proteinase K treatment of samples was carried out as follows. After the import incubations, two 90-μl aliquots of respective reaction mixtures were removed and diluted to 390 μl with SHKCl Buffer (600 mM sorbitol, 50 mM Hepes, pH 7.2, 80 mM KCl). One diluted sample was treated with proteinase K (100 μg/ml), while the other was untreated. Both were then incubated on ice for 30 min. Next, phenylmethylsulfonyl fluoride (0.5 mM) was added to each sample and mitochondria were reisolated. Analysis for import by SDS-polyacrylamide gel electrophoresis and fluorography and laser densitometry of x-ray films were as previously described (Cyr and Douglas, 1991).

Protein Import into ΔATP1 Mitochondria—ΔATP1 mitochondria (200 μg/ml) were preincubated in import buffer (see above) for 3 min at 25 °C to promote turnover of matrix ATP. Then, as indicated, carboxyatractyloside (5 μM), NADH (2 mM), and ATP (2 mM) were added in successive order, with each addition being followed by a 3-min incubation period at 25 °C. Reactions were then started by addition of precursor protein in reticulocyte (0.5%, v/v). At reticulocyte lysate concentrations below 1%

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¶ To whom correspondence should be addressed. Tel.: 49-89-5996-312; Fax: 49-89-5996-270.

[1] The abbreviations used are: hsp70, 70-kDa heat shock protein; mthsp, mitochondrial heat shock protein.

[2] R. A. Stuart, A. Gruhler, I. van der Klei, B. Guiard, H. Koll, and W. Neupert, submitted for publication.

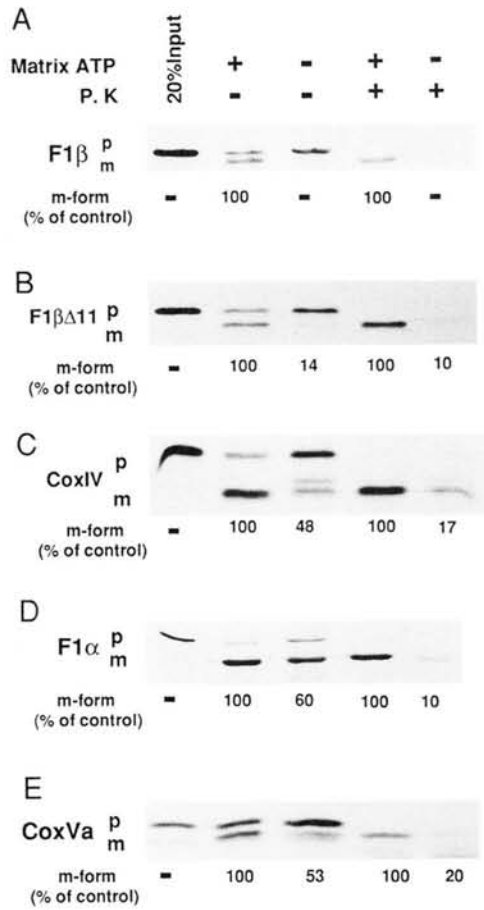

FIG. 1. **Depletion of matrix ATP inhibits the processing and import of several mitochondrial precursor proteins.** Isolated mitochondria (40 μg) and various ^{35}S-labeled precursor proteins were incubated in 200-μl reaction mixtures for 5 min at 25 °C and assayed for processing and import as described under "Experimental Procedures." – *Matrix ATP* denotes that mitochondria were treated with carboxyatractyloside and oligomycin to deplete matrix ATP levels prior to import incubations. + *Matrix ATP* denotes mitochondria that were mock-treated prior to import incubations. + *P.K* denotes that mitochondria were treated with proteinase K after import reactions were carried out (see "Experimental Procedures" for details). 100% of control import for $pF_1\beta$, $p\Delta\beta11$, pCoxIV, $pF_1\alpha$ and pCoxVa represented 15, 20, 31, 53, and 20% of input, respectively. Designators are as follows: $pF_1\beta$, F_1-ATPase β subunit precursor; $pF_1\beta\Delta11$, a $F_1\beta$ construct in which amino acids 123–381 were internally deleted; pCoxIV, cytochrome oxidase subunit IV precursor; pCoxVa, cytochrome oxidase subunit Va precursor.

(v/v), uptake of lysate ATP by ΔATP1 mitochondria is insufficient to stimulate protein import.[3]

RESULTS

A Novel Import Intermediate Accumulates when Matrix ATP Is Limiting for Protein Translocation across the Inner Membrane—The presequence is removed from precursor proteins upon translocation into and exposure of its cleavage site in the matrix (Rassow *et al.*, 1990). The maturation of precursor proteins can be used as an indicator of stable transfer of the complete presequence into the matrix. To determine whether matrix ATP plays a specific role in presequence translocation across the inner membrane, ATP levels were lowered to reduce mthsp70 activity and the processing and import of five different precursor proteins were examined. Reduction of matrix ATP severely retarded the processing and import of all precursors tested (Fig. 1, *A–E*). Processing and import of $pF_1\beta$ were completely inhibited (Fig. 1*A*). Processing and import of $pF_1\beta\Delta11$, an $F_1\beta$ construct that is imported at low cytosolic ATP (Chen and Douglas, 1988), were inhibited by about 90% (Fig. 1*B*). Processing of pCoxIV, $pF_1\alpha$, and pCoxVa was inhibited by 40–52% and import by 80–90% (Fig. 1, *C–E*). The small quantity of mature protein which was resistant to protease that formed from the different precursors when matrix ATP levels were low was most likely imported into the matrix (Fig. 1). However, it is also possible that under these experimental conditions, import of the different precursors was arrested after translocation across the outer membrane and the mature proteins formed are stuck in the intermembrane space (Hwang *et al.*, 1991).

When mitochondria isolated from temperature-sensitive mthsp70 (ssc1) mutants were preincubated at the non-permissive temperature prior to import, inhibition of both precursor processing and import occurred.[3] The severity of this inhibition was similar to that observed when matrix ATP was depleted (Fig. 1). Thus, matrix ATP depletion, apparently reflecting the inactivation of mthsp70, reduces the extent to which the complete presequence is stably translocated across the inner membrane into the matrix.

In order to further analyze the role of matrix ATP in transfer of presequences across the inner membrane, we thought it necessary to be able to reversibly modulate matrix ATP levels. Thus, we studied import with mitochondria that were unable to produce ATP via oxidative phosphorylation. Mitochondria from yeast strain W303aΔATP1, which lack a functional ATP synthase but can take up ATP from the reaction media via the ATP/ADP carrier to supply matrix ATP consuming reactions, were employed in our next experiments.

ΔATP1 mitochondria process and import $pF_1\alpha$ at efficiencies comparable to wild type mitochondria when incubated in the presence of ATP (Fig. 2, *A* and *B*). Efficient protein translocation into ΔATP1 mitochondria was dependent on uptake of exogenous ATP into the matrix; pretreatment of mitochondria with carboxyatractyloside prior to ATP addition (to block the ATP/ADP carrier) severely inhibited both processing and import of $pF_1\alpha$ (Fig. 3*A*, compare *lanes 2* and *3*). Similarly, when ATP was omitted from reaction mixtures, the rate of $pF_1\alpha$ processing was reduced by 75% and protease sensitive $pF_1\alpha$ accumulated with mitochondria (Fig. 2, *A* and *C*). The small amount of $mF_1\alpha$ formed under these conditions was not translocated into the matrix, but spanned the mitochondrial membranes, since it could be completely digested when intact mitochondria were incubated with proteinase K (Fig. 2, *A* and *C*). The unlikely possibility that inhibition of presequence processing was due to reduction of the $\Delta\psi$ across the inner membrane of ΔATP1 mitochondria when ATP was omitted was ruled out by direct measurements of $\Delta\psi$. Components of the import buffer maintained $\Delta\psi$ unaltered when "ATP1 mitochondria" were incubated in the presence or absence of ATP.[3]

Both ATP-depleted mitochondria and ΔATP1 mitochondria require matrix ATP for efficient presequence processing (Figs. 1 and 2). When matrix ATP is insufficient to drive protein translocation, stable translocation of the presequence across the inner membrane is apparently reduced (Fig. 1 and Fig. 2, *A* and *C*). The reduced efficiency of this reaction correlated with the time-dependent association with mitochondria of an unprocessed precursor that appeared to be a novel import intermediate (Fig. 2, *A* and *C*).

$\Delta\psi$ and ATP Are Required for Completion of Import of the Novel Import Intermediate—To determine whether the $pF_1\alpha$ that accumulated with ΔATP1 mitochondria represented an authentic import intermediate, its further import into the matrix was characterized. ΔATP1 mitochondria were subjected to two sequential incubations termed primary and chase (Fig. 3*A*). In the primary incubation, $pF_1\alpha$ was allowed to accumulate with ΔATP1 mitochondria, which were incubated with NADH but without added ATP ($\Delta\psi$ present, low matrix ATP;

[3] D. M. Cyr, R. A. Stuart, and W. Neupert, unpublished observation.

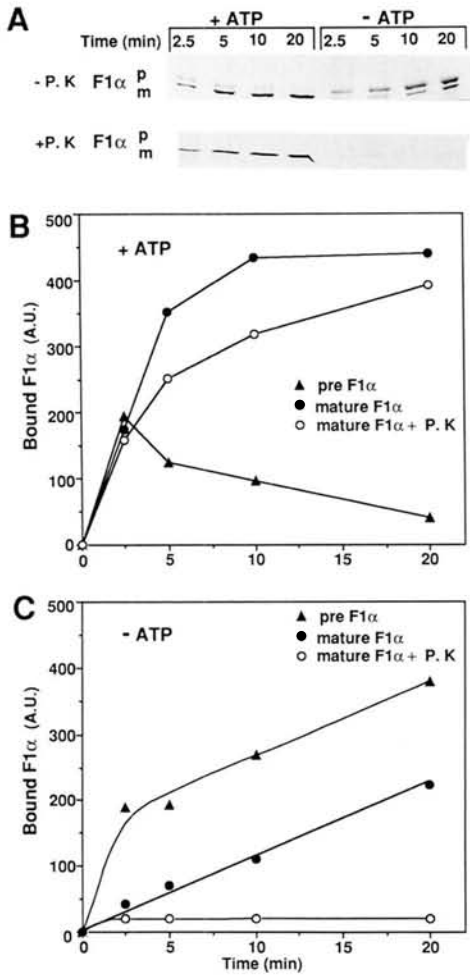

FIG. 2. **Time course of protein import into mitochondria from the ΔATP1 yeast strain.** Isolated ΔATP1 mitochondria (200 μg) were incubated in import buffer (1.0 ml), with NADH (2 mM), ATP (1 mM, as indicated), and $F_1\alpha$ precursor (0.5% lysate) at 25 °C. At indicated times, 200-μl aliquots were removed and transferred to tubes, made 1.8 μM in valinomycin, and placed on ice to stop import. At this point, samples were analyzed for processing and import as described under "Experimental Procedures." + *ATP*, 45% of input was processed and imported at the 10-min time point. – *ATP*, approximately 11% of input was processed after 10 min of incubation, but only 2.2% of input was translocated to a protease-protected space. *B* and *C*, quantitation of Fig. 3*A* expressed in arbitrary units (*A.U.*).

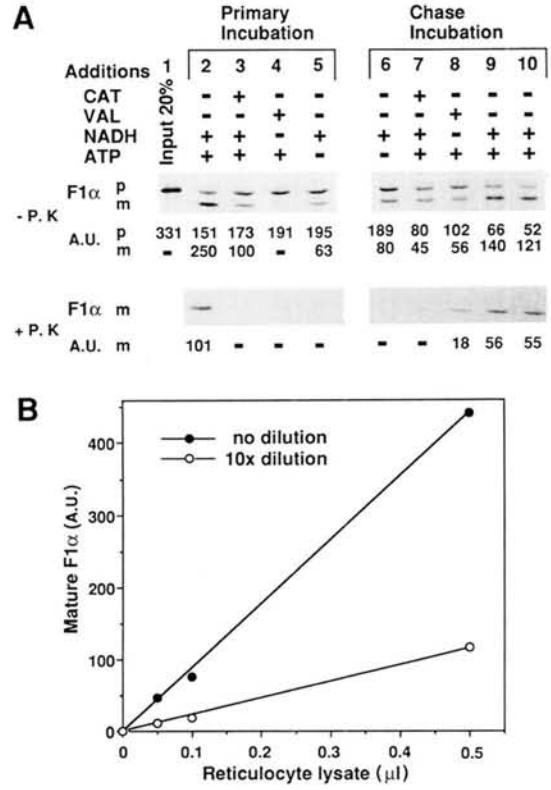

FIG. 3. **Import intermediates that associate with mitochondria can be chased into the matrix.** *A*, in a primary reaction (*lanes 1–5*), ΔATP1 mitochondria (20 μg) and $pF_1\alpha$ in reticulocyte (0.5%) were incubated in import buffer (100 μl) for 5 min at 25 °C with the indicated additions. Reactions were then placed on ice and divided into 45-μl aliquots and treated with proteinase K. In a secondary reaction (chase; *lanes 6–10*), ΔATP1 mitochondria that had been incubated with $pF_1\alpha$ and NADH (2 mM) for 5 min at 25 °C (*lane 5*) were placed on ice, reisolated, and resuspended in fresh ice-cold import buffer (100 μl). Indicated additions were made, and chase reactions were carried out at 25 °C. After a 10-min incubation period, reactions were cooled to 0 °C and treated with proteinase K as described above. In *lane 10*, the volume of the chase reaction was increased from 100 μl to 1000 μl. *CAT* denotes carboxyatractyloside; *VAL* denotes valinomycin, which when present was 1.8 μM. *A.U.*, arbitrary units. *B*, dilution control experiment. $pF_1\alpha$ in reticulocyte (as indicated) was incubated with mock-treated ΔATP1 mitochondria (20 μg) in 100- or 1000-μl reaction mixtures containing ATP (1 mM) and NADH (2 mM) for 10 min at 25 °C. Import of $F_1\alpha$ was then assayed and quantitated. Mock-treated mitochondria were incubated exactly as described for the chase reactions (above) except cold reticulocyte lysate was substituted for $pF_1\alpha$-containing lysate in the primary reaction.

Fig. 3*A*, *lane 5*). Mitochondria were then reisolated, resuspended in import buffer and subjected to chase incubation. Chase incubation in the presence of ATP and $\Delta\psi$ led to the processing and import of a major portion of bound $pF_1\alpha$ (Fig. 3*A*, compare *lanes 5* and *9*, ± *P.K*). The efficiency of chase import was high, since import in this two-step reaction represented approximately 60% of that observed in the single step import reaction (Fig. 3*A*, compare *lanes 2* and *9*, + *P.K*). Chase incubation in the presence of $\Delta\psi$ alone or $\Delta\psi$ in combination with carboxyatractyloside and ATP did not lead to precursor processing or import (Fig. 3*A*, *lanes 6* and *7*, ± *P.K*). To demonstrate that the secondary import of $pF_1\alpha$ represents completion of import by a bound intermediate, ΔATP1 mitochondria were diluted 10-fold prior to the chase incubation. This had no significant influence on the completion of $pF_1\alpha$ import (Fig. 3*A*, compare *lanes 9* and *10*). In contrast, when $pF_1\alpha$ in reticulocyte lysate and ΔATP1 mitochondria were diluted 10-fold, import was inhibited by 80% (Fig. 3*B*). Thus, $pF_1\alpha$ associated with ΔATP1 mitochondria that are low in matrix ATP represents a true import intermediate. The $\Delta\psi$ requirement for further movement of this intermediate along the mitochondrial import pathway supports the conclusion that a step dependent on matrix ATP is required for efficient presequence translocation across the inner membrane.

In these same experiments, we also observed a minor import intermediate that was processed and protease-sensitive and that required only matrix ATP for further import (Fig. 3*A*, compare *lanes 5* and *8*). Thus when matrix ATP levels are low, presequence translocation across the inner membrane occurs, but at low efficiency.

In a previous study, matrix ATP depletion caused an import intermediate of a CoxIV-dihydrofolate reductase fusion protein to associate with mitochondria. This intermediate was unprocessed, resistant to protease digestion and required only ATP for further translocation into the matrix (Hwang *et al.*, 1991). Since it was beyond the $\Delta\psi$-dependent import step, this intermediate appears similar to the minor intermediate observed above, except the carboxyl terminus of the protein has already crossed the outer membrane (Hwang *et al.*, 1991). The observation that the CoxIV-dihydrofolate reductase intermediate

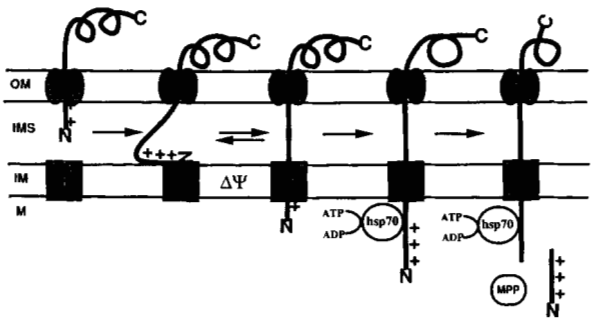

FIG. 4. **A model for the role of Δψ, matrix ATP, and mitochondrial hsp70 in presequence translocation into the mitochondrial matrix.** Positive charges on the precursor protein denote the presequence. *MPP* denotes the matrix processing protease. Other abbreviations are as follows: *OM*, outer membrane; *IMS*, intermembrane space; *IM*, inner membrane; *M*, matrix. *Ovals* and *rectangles* denote the protein translocation apparatus.

was unprocessed indicates that a step dependent on matrix-ATP is also required to assist in the translocation of the complete presequence of this precursor across the inner membrane.

DISCUSSION

We have characterized a novel translocation intermediate that accumulates before the Δψ-dependent import step. This is a true intermediate on the mitochondrial assembly pathway because it can be further translocated from the membrane into the matrix. This intermediate accumulates when matrix ATP levels are too low to support mthsp70-dependent protein translocation into the matrix and Δψ is sufficient for presequence translocation across the inner membrane. The identification of this new import intermediate indicates that efficient translocation of the presequence across the inner membrane requires a step dependent on matrix ATP. We propose that presequence translocation across the inner membrane is reversible and requires the action of mthsp70 (Fig. 4). In this concerted mechanism Δψ drives translocation of the presequence across the inner membrane, while mthsp70 serves to increase the efficiency of this reaction by binding the precursor and stabilizing the presequence on the matrix side (Fig. 4). This could occur through direct interaction of mthsp70 with the presequence or with mature regions of the precursor.

This model predicts that the matrix ATP requirement for presequence processing will vary with the stability and depth to which the presequence is initially inserted across the inner membrane. These parameters are likely to be determined by the length of the presequence and conformation of the precursor at the time of translocation. Indeed, we find that the processing of the different precursors tested in this study exhibits differential sensitivity to depletion of matrix ATP. Precursor conformation obviously plays a role in determining the ATP requirement for processing; when matrix ATP was depleted, $F_1\beta\Delta11$, a construct of $pF_1\beta$ that lacks an internal homotetramer binding site (Chen and Douglas, 1988), is processed with higher efficiency than $pF_1\beta$. Similarly, precytochrome b_2 constructs, which lack a heme binding domain near the amino terminus, are processed with higher efficiency by mitochondria depleted of matrix ATP than authentic precursor.[2] It appears that presequence processing and protein translocation into the matrix also have different ATP requirements. Presequence processing is less sensitive to matrix ATP depletion, probably because the reaction is only made more efficient by matrix ATP and/or requires only one round of ATP-dependent binding and release by mthsp70. Protein translocation into the matrix could be more sensitive to matrix ATP depletion because several cycles of mthsp70 action appear to be required (Neupert *et al.*, 1990).

Inactivation of mthsp70 to varying degrees appears to result in inhibition of different steps of the mitochondrial import pathway. In studies with mitochondria isolated from a temperature-sensitive mthsp70 mutant strain termed ssc1-2, precursor processing was normal, but protein translocation into the matrix was inhibited (Kang *et al.*, 1990). However, in studies with mitochondria isolated from a different temperature-sensitive mthsp70 mutant strain termed ssc1–3, defects in both precursor processing and import were observed.[3,4] Similarly, experimental conditions that reduce the concentration of matrix ATP to different extents may also give rise to the accumulation of import intermediates at different stages of the mitochondrial assembly pathway. This is a possible explanation for the observation that, in this study, we observed the accumulation of an unprocessed and protease sensitive import intermediate when matrix ATP levels are low, whereas in a study that employed different experimental conditions to reduce matrix-ATP levels, an unprocessed but protease-insensitive translocation intermediate accumulated (Hwang *et al.*, 1991).

It was recently reported that import and sorting of pCoxVa to the inner membrane of ssc1-2 mitochondria are normal at the non-permissive temperature (Miller and Cumsky, 1993). This result suggested that CoxVa biogenesis is independent of matrix ATP (Miller and Cumsky, 1993). In the present study, efficient processing and import of pCoxVa were found to require matrix ATP. The probable cause for these differing results is that matrix ATP depletion leads to reduction of mthhsp70 activity to a level which is lower than that of mthsp70 in mitochondria from the ssc1-2 mutant. It remains to be tested whether low levels of mthsp70 activity are generally required for import and sorting of inner membrane proteins as compared to matrix proteins.

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