Energy Requirements for Unfolding and Membrane Translocation of Precursor Proteins during Import into Mitochondria*

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ATP is involved in conferring transport competence to numerous mitochondrial precursor proteins in the cytosol. Unfolded precursor proteins were found not to require ATP for import into mitochondria, suggesting a role of ATP in the unfolding of precursors. Here we report the unexpected finding that a hybrid protein containing the tightly folded passenger protein dihydrofolate reductase becomes unfolded and specifically translocated across the mitochondrial membranes independently of added ATP. Moreover, interaction of the precursor with the mitochondrial receptor components does not require ATP. The results suggest that ATP is not involved in the actual process of unfolding during membrane translocation of precursors. ATP rather appears to be necessary for preventing the formation of improper structures in precursors in the cytosol and for folding of imported polypeptides (and release from) chaperone-like molecules in the mitochondrial matrix.

Folding and unfolding of precursor proteins during membrane translocation are essential reactions of the complex pathway proteins take to traverse biological membranes. So far very little is known about the energetic aspects of these reactions. Transport of precursor proteins into various cell organelles was found to depend on the addition of ATP (1–3). Studies on protein transport into mitochondria and the endoplasmic reticulum suggested that ATP is involved in conferring a transport-competent conformation to the precursor proteins in the cytosol (2, 4–6). Incompletely synthesized forms did not depend on ATP for import, whereas import of mitochondrial precursor protein) was not inhibited by removal of ATP. Specifically, the interaction with the recently discovered import receptor MOM19 (15), unfolding, and membrane translocation of the precursor protein did not require ATP. We conclude that ATP is not directly involved in the transfer of precursor proteins across the mitochondrial membranes. ATP may rather be involved in the maintenance of transport competence of those precursor proteins that might form improper structures in the cytosol.

MATERIALS AND METHODS

The following methods were employed: growth of Neurospora crassa (wild-type 74A) (16); isolation of mitochondria (13, 17); construction of hybrid proteins and other DNA manipulations (13, 18–20); synthesis of precursor proteins in rabbit reticulocyte lysates in the presence of [35S]methionine, ATP, and an ATP-regenerating system (13, 21) by coupled transcription/translation (22); treatment of reticulocyte lysate and of mitochondria with apyrase (23) at 25 or 0 °C (control reactions received an equivalent amount of a heat (95 °C)-inactivated apyrase preparation); binding of IgGs directed against outer membrane proteins to isolated mitochondria (15); import of precursor proteins into isolated mitochondria and treatment with protease (13, 24); sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25); fluorography (26); quantitation of the fluorographs by laser densitometry (20) using a calibration curve.

The import assays contained 20–50% (v/v) reticulocyte lysate, potassium ascorbate (8 mM), N,N,N',N'-tetramethylphenylenediamine (0.2 mM), antimycin A (8 μM), oligomycin (20 μM), N. crassa mitochondria (10 μg of mitochondrial protein), and BSA buffer (250

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1 The abbreviations used are: DHFR, dihydrofolate reductase; BSA, bovine serum albumin; b(1-x)-DHFR, hybrid protein between x amino-terminal amino acid residues of the precursor of cytochrome b(1) and entire DHFR; F(0), F(0)-F(1)-ATPase subunit α; F(0), F(1)-ATPase subunit β; Iasp60, heat shock protein of 60 kDa; Iasp70, heat shock protein of 70 kDa; MOMP18, mitochondrial outer membrane protein of 19 kDa, p-, i-, m-, precursor-, intermediate-, and mature-sized forms of a protein, respectively; MOPS, 3-(N-morpholino)propanesulfonic acid.

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Role of ATP in Mitochondrial Protein Import

RESULTS

ATP Dependence of Mitochondrial Import of Cytochrome b$_2$-DHFR Hybrid Proteins—We investigated the ATP dependence of import into mitochondria of three hybrid proteins composed of portions of the precursor of cytochrome b$_2$ (amino terminus) and the entire DHFR (carboxyl terminus). The hybrid proteins contained the 167, 331, or 561 amino-terminal amino acid residues of the cytochrome b$_2$ precursor polypeptide (Fig. 1A). The cell free import system, isolated N. crassa mitochondria and rabbit reticulocyte lysate containing the [%sup]35S [%sup]methionine-labeled precursor proteins, was depleted of ATP by preincubation with apyrase. Oligomycin was included to prevent synthesis of ATP by the F$_0$F$_1$-ATPase. Pretreatment with apyrase, an ATPase and ADPase from potato, had been found to inhibit mitochondrial import of many precursor proteins, e.g. F$_1$-ATPase subunit β (F$_1$β), and this inhibition could be reversed by readdition of ATP but not by the addition of nonhydrolysable ATP analogues (19, 23, 27, 28). Import of the hybrid protein b$_2$(1-561)-DHFR was inhibited to the same extent as import of F$_1$β by depletion of ATP, while inhibition of import of b$_2$(1-331)-DHFR was less pronounced (Fig. 1B). Import of b$_2$(1-167)-DHFR was practically not affected by the pretreatment with apyrase (Fig. 1B). It appeared that the shorter the cytochrome b$_2$ portion of the hybrid protein was, the less ATP was required for import.

We recently reported that unfolding of the DHFR domain of b$_2$(1-167)-DHFR on the mitochondrial surface was a prerequisite for its translocation into mitochondria (13). This raised the interesting possibility that unfolding and membrane translocation of the DHFR part did not require ATP. Unfolding and Membrane Translocation of DHFR Domain Are Independent of Added ATP—We first investigated if under the conditions used the DHFR domain of the hybrid protein b$_2$(1-167)-DHFR was tightly folded. This can be assessed by probing its resistance to relatively high concentrations of proteinase K (13). Treatment of the precursor of b$_2$(1-167)-DHFR with proteinase K produced a fragment that was slightly larger than authentic DHFR (13) and was resistant to all concentrations of proteinase K tested (Fig. 2A). Similarly, when b$_2$(1-167)-DHFR was accumulated in contact sites of outer and inner membranes (see below) by performing import at a low temperature (8°C), treatment with proteinase K generated a fragment of high protease resistance (Fig. 2C).

Translocation of b$_2$(1-167)-DHFR across the mitochondrial membranes can be experimentally divided into two steps (13). First, the cytochrome b$_2$ portion is inserted into contact sites (10); the presequence is proteolytically processed by the processing peptidase in the mitochondrial matrix (29) while the DHFR domain remains on the cytosolic side. In a second step, the DHFR is unfolded on the mitochondrial surface and translocated across the membranes. In the experiment described in Fig. 3, the ATP dependence of these two import steps of b$_2$(1-167)-DHFR was analyzed. Translocation of b$_2$(1-167)-DHFR into contact sites performed at 8°C was only slightly inhibited by the pretreatment with apyrase (Fig. 3A). For comparison, insertion of the precursor of F$_1$β into contact sites strongly depended on the presence of ATP (Fig. 3A) (23, 27, 30). For the second step, b$_2$(1-167)-DHFR was first accumulated in contact sites (at 8°C), and then the mitochondria were treated with apyrase and warmed to 25°C to allow completion of import ("chase"). Treatment with apyrase did not inhibit the completion of translocation of b$_2$(1-167)-DHFR (Fig. 3B). In contrast, completion of import of F$_1$β strongly depended on the presence of ATP, i.e. was inhibited by the treatment with apyrase (Fig. 3B) (27, 31).
this possibility we asked if by(l-167)-DHFR uses the import preimmune sera, did not inhibit. We conclude that b(l-167)-port site which allows ATP-independent import. To exclude proteins, because it might use a different mitochondrial import. For slightly larger than authentic DHFR (in addition, at low concentrations, the different number of methionines in precursor and fragment was taken into consideration. b2(1-167)-DHFR accumulated in contact sites. The experiment was performed as described in the legend of Fig. 1B except that the pretreatment with apyrase was omitted and that the concentration of BSA was 1% (w/v). p-bz(l-167)-DHFR for A and in Ref. 13. Treatment with proteinase K produced the fragment slightly larger than authentic DHFR (in addition, at low concentrations of protease a fragment of about 30 kDa was observed) (13). For quantitation of p-b(l-167)-DHFR and the DHFR-containing fragments, the different number of methionines in precursor and fragment was taken into consideration. B, b2(1-167)-DHFR accumulated in contact sites. The experiment was performed as described in the legend of Fig. 1B except that the pretreatment with apyrase was omitted and that the import reaction was incubated for 20 min at 8 °C. The mitochondria containing b2(1-167)-DHFR accumulated in contact sites were resuspended in BSA buffer (containing 1% BSA) in the presence of valinomycin (0.5 μM), antimycin A, and oligomycin, and treated with proteinase K for 20 min at 0 °C. Mitochondria and supernatant were separated, and the amounts of i-b2(1-167)-DHFR (in the mitochondrial fraction) and the DHFR-containing fragment (in the supernatant) were analyzed as described for A and in Ref. 13. Treatment with proteinase K produced the fragment slightly larger than authentic DHFR (13).

Receptor MOM19—Import of b2(1-167)-DHFR could be independent of added ATP, in contrast to many other precursor proteins, because it might use a different mitochondrial import site which allows ATP-independent import. To exclude this possibility we asked if b2(1-167)-DHFR uses the import receptor MOM19 that was recently shown to function as a receptor for most mitochondrial precursor proteins studied, including the precursor of Foβ (15). IgGs directed against MOM19 were bound to mitochondria and import of b2(1-167)-DHFR was tested. Fig. 4 shows that IgGs against MOM19 strongly inhibited import whereas control IgGs, against the major outer membrane protein porin or from antimycin A, did not inhibit. We conclude that b2(1-167)-DHFR employs the receptor MOM19, leading to the interesting notion that binding of a precursor to and release from MOM19 appear to be ATP-independent. The conclusion that b2(1-167)-DHFR uses the same mitochondrial import site as ATP-dependent precursor proteins is further supported by the finding that b2(1-167)-DHFR accumulated in contact sites inhibits import of other precursor proteins such as Foβ, indicating that the precursors use the same translocation contact sites (13).

In summary, a precursor protein with a tightly folded carboxyl-terminal domain can be imported into mitochondria although the ATP levels were drastically reduced. Moreover, unfolding and membrane translocation of the DHFR portion itself was found to be independent of added ATP. Although involvement of bound ATP (that may not be hydrolyzed by apyrase) cannot be excluded, the behavior of b2(1-167)-DHFR in the in vitro import reaction is in clear contrast to that of several other mitochondrial precursor proteins where the degree of unfolding required for import appeared to correlate with the amounts of ATP necessary (7, 8, 27). In all likelihood, ATP is thus not generally involved in receptor binding, unfolding, and membrane translocation of proteins during import into mitochondria. This conclusion is supported by results that were previously obtained with import of the precursor of Fo-ATPase subunit 9 (Fo9). The precursor of Fo9

![Figure 2](http://www.jbc.org/)

**Fig. 2.** The DHFR domain of b2(1-167)DHFR is in a folded conformation. A, b2(1-167)-DHFR synthesized in vitro. Reticulocyte lysate containing radiolabeled b2(1-167)- DHFR was treated with proteinase K for 20 min at 0 °C under standard conditions (see “Materials and Methods”) except that mitochondria were omitted and that the concentration of BSA was 1% (w/v), p-b2(1-167)-DHFR and the fragments were quantified by laser densitometry of the fluorographs. Treatment with proteinase K produced a fragment slightly larger than authentic DHFR (in addition, at low concentrations of protease a fragment of about 30 kDa was observed) (13). For quantitation of p-b2(1-167)-DHFR and the DHFR-containing fragments, the different number of methionines in precursor and fragment was taken into consideration. B, b2(1-167)-DHFR accumulated in contact sites. The experiment was performed as described in the legend of Fig. 1B except that the pretreatment with apyrase was omitted and that the concentration of BSA was 1% (w/v). p-bz(l-167)-DHFR for A and in Ref. 13. Treatment with proteinase K produced the fragment slightly larger than authentic DHFR (13).

![Figure 3](http://www.jbc.org/)

**Fig. 3.** ATP dependence of distinct steps in import of b2(1-167)-DHFR. A, transport into contact sites. The experiment was performed as described in the legend of Fig. 1B except that the import reaction was incubated for 20 min at 8 °C and the treatment with proteinase K was omitted. In control samples, 92% of i-b2(1-167)-DHFR and 88% of m-Foβ were accessible to externally added proteinase K, i.e. were spanning mitochondrial contact sites (10, 13, 48, 49). B, completion of transport into mitochondria. Accumulation of precursor proteins in contact sites was performed as described for A (the pretreatment with apyrase was omitted). Then valinomycin (0.5 μM) was added to dissipate the mitochondrial membrane potential (thereby insertion of precursor proteins into contact sites is prevented whereas completion of translocation from contact sites into the matrix is possible (10, 49)). The reactions were incubated with apyrase (20 units/ml) or a heat-inactivated apyrase preparation for 20 min at 0 °C. After incubation at 25 °C for the indicated time periods ("chase"), treatment with proteinase K (30 μg/ml) was performed.
Role of ATP in Mitochondrial Protein Import

We describe the seemingly paradoxical situation that both tightly folded precursor proteins (as in the experiments with hsp70) and unfolded precursor proteins (8, 9) do not require ATP for import into mitochondria. Moreover, both a stably folded precursor protein and an unfolded precursor protein had been pretreated with trypsin (15 μg/ml) (bypass import (50)), representing about 15% of the total import, was subtracted (15). Monospecific IgGs against more than 15 other outer membrane proteins of N. crassa mitochondria (15) did not inhibit import (data not shown). The similar result was obtained when the import system was depleted of ATP (as described in the legend of Fig. 1B) prior to the preincubation with IgGs. Furthermore, the amount of bypass import did not depend on the levels of ATP in the import reaction.

Fig. 4. b2(1–167)-DHFR uses the import receptor MOM19.

The experiment was performed as described in the legend of Fig. 1B with the following modifications. The pretreatment with apyrase and the addition of antimycin A and oligomycin were omitted; the mitochondria were preincubated with the indicated IgGs as described (15). The amount of i-b2(1–167)-DHFR imported into mitochondria that had been pretreated with trypsin (15 μg/ml) (bypass import (50)), representing about 15% of the total import, was subtracted (15). The experiment was performed as described in the legend of Fig. 1B

DISCUSSION

What is ATP then doing? It is suggested that cofactors bind to folding intermediates that expose certain critical features such as hydrophobic (36) or certain hydrophilic segments (37). These complexes may not be competent for translocation because the tight interactions cannot be relieved by the unfolding process during translocation. ATP would be required for allowing the dissociation of precursors and cofactors in the course of translocation. Folded proteins would not bind cofactors and thus would not need ATP for releasing them. This view is consistent with the general role thought to be played by 70-kDa stress proteins that act in an ATP-dependent manner, namely binding to not fully folded precursors in order to prevent the formation of improper conformations or interactions (36, 38, 39). As the energy requirement for complete unfolding of many proteins is as low as 5–10 kcal/mol (40), it is well conceivable that the unfolding of correctly folded polypeptide chains can be performed by the mitochondrial import machinery without the need for ATP as external energy source. Moreover, we conclude that interaction of precursor proteins with the membrane-bound components of the mitochondrial import machinery, such as binding to and release from the receptor MOM19 (15) as well as translocation into and through contact sites (13), does not require the addition of ATP.

The observations made here cast new light on a number of results reported previously. A hybrid protein between the presequence of cytochrome oxidase subunit IV and DHFR was unfolded on the mitochondrial surface in the absence of added ATP (41). However, one of the further import steps of this hybrid protein, i.e. membrane translocation, proteolytic processing, or (re)folding in the matrix, required ATP in the mitochondrial matrix (41, 42). This led to the conclusion that the ATP-requiring step assumed to occur in the cytosol would in fact take place in the matrix and may be necessary for membrane translocation. In view of the results reported here, it seems possible that the subunit IV-DHFR hybrid protein bypassed the ATP-dependent mechanism in the cytosol due to a correctly folded structure. With regard to the ATP requirement in the matrix, we found for a number of precursor proteins imported into the matrix that interaction with the heat shock protein hsp60 in an ATP-dependent manner represents an essential step for (re)folding and assembly of the proteins and can affect the rates of proteolytic processing of precursors (9, 43). The ATP-dependent step of import of the subunit IV-DHFR hybrid protein thus may well be related to interaction with the “chaperonin” hsp60 (38). The hybrid protein b2(1–167)-DHFR indeed interacts with hsp60 in a

1 R. Pfanner, N. Pfanner, and W. Neupert, unpublished data

2 R. Pfaller, N. Pfanner, and W. Neupert, unpublished data
reaction involving ATP hydrolysis; this step is not directly necessary for membrane translocation. Moreover, recent studies suggest that a hsp70 in the mitochondrial matrix, termed necessary for membrane translocation. ATP hydrolysis might be required for release of the precursors from Ssclp (36, 38, 39), setting the chaperone free for new rounds of import. It is unknown if ATP that could be tightly bound to Ssclp (and would thus not require functional hsp60 for intramitochondrial sorting). ATP hydrolysis might be required for release of the precursors from Ssclp (36, 38, 39), setting the chaperone free for new rounds of import. It is unknown if ATP that could be tightly bound to Ssclp (and would thus not require functional hsp60 for intramitochondrial sorting). ATP hydrolysis might be required for release of the precursors from Ssclp (36, 38, 39), setting the chaperone free for new rounds of import. It is unknown if ATP that could be tightly bound to Ssclp (and would thus not require functional hsp60 for intramitochondrial sorting).

We propose a model (Table I) where at least two ATP-dependent steps exist in import and assembly of mitochondrial precursor proteins: (i) maintenance or conferring of a transport-competent conformation of the cytosolic side; and (ii) intramitochondrial (re)folding and sorting of precursor proteins, including "recycling" of chaperone-like components in the matrix. Both of these ATP-dependent reactions can be bypassed: the first step by artificially unfolded precursor proteins (8, 9) or by tightly folded precursor proteins (this study); the second step by the ADP/ATP carrier (27) which does not require functional hsp60 for intramitochondrial folding and sorting (40). The requirement for a certain factor (ATP) at multiple steps illustrates the complexity of mitochondrial protein import and cautions against the use of minimal models.

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| Import step | Location | Putative mechanism and/or component(s) involved | Free ATP required | Example(s) |
|-------------|----------|---------------------------------------------|------------------|------------|
| I. Maintenance or conferring of transport competence of precursors | Outside mitochondrion | (i) Association of precursors with cytosolic protein factors or (ii) native folding of precursor domain(s) or (iii) artificial unfolding of precursors | + | AAC, porin, F_6, cytochromes b_{1,2} and c_{1,2} |
| II. Extensive unfolding and membrane translocation of precursors | Mitochondrial contact sites | Performed by membrane-bound import machinery | - | All precursors transported to inner membrane or matrix |
| III. Sorting and folding of imported proteins | Mitochondrial matrix/inner membrane | (i) Via hsp60 or (ii) without hsp60 | + | F_6, F_7, Fe/S protein, cytochromes b_{1,2} and c_{1,2} b_{1,2}(1-167)-DHFR |

TABLE I
Hypothetical scheme of ATP-dependent and ATP-independent reactions in import of precursor proteins into mitochondria
Role of ATP in Mitochondrial Protein Import

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