

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

(Received for publication, April 20, 1990)

Nikolaus Pfanner, Joachim Rassow, Bernard Guiard‡, Thomas Söllner, Franz-Ulrich Hartl, and Walter Neupert

From the Institut für Physiologische Chemie, Universität München, Goethestrasse 33, D-8000 München 2, Federal Republic of Germany and the ‡Centre de Génétique Moléculaire, Laboratoire propre du Centre National de la Recherche Scientifique associé à l'Université Pierre et Marie Curie, Gif-sur-Yvette 91190, France

**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 of precursors in the cytosol and for folding of imported polypeptides on (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 and thus loosely folded mitochondrial polypeptide chains required less ATP for import than the corresponding completed precursor proteins (7). Artificially unfolded precursor forms did not depend on ATP for import, whereas import of the (partially folded) authentic precursors required ATP (8, 9). As membrane translocation of mitochondrial precursor proteins requires a loosely folded conformation of the polypeptide chain (10-13), it was assumed that ATP participates in modulating the folding state of precursor proteins.

Two major possible roles of ATP can be envisaged. ATP could be directly involved in the process of unfolding of precursor proteins. *e.g.* via ATP-dependent cytosolic cofactors (14). Alternatively, ATP (and cytosolic cofactors) could be required to prevent misfolding or aggregation of precursor

proteins that cannot be reversed by the membrane-associated import machinery of mitochondria. In the first case, the levels of ATP required for import should correlate with the degree of folding of a polypeptide chain, *i.e.* a precursor protein with a stably folded structure should strongly depend on ATP for import. In the second case, a precursor protein with a stably yet correctly folded domain may be independent of ATP for import.

To distinguish between these possibilities, we investigated mitochondrial import of several hybrid proteins containing the correctly and tightly folded dihydrofolate reductase (DHFR)<sup>1</sup> polypeptide at the carboxyl terminus. We found that import of one hybrid protein with a relatively short amino-terminal portion (that was derived from a 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 [<sup>35</sup>S]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 20-30% (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

\* This study was supported by the Sonderforschungsbereich 184 and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> The abbreviations used are: DHFR, dihydrofolate reductase; BSA, bovine serum albumin; *b*<sub>2</sub>(1-x)-DHFR, hybrid protein between *x* amino-terminal amino acid residues of the precursor of cytochrome *b*<sub>2</sub> and entire DHFR; F<sub>0</sub>9, F<sub>0</sub>F<sub>1</sub>-ATPase subunit 9; F<sub>1</sub>β, F<sub>0</sub>F<sub>1</sub>-ATPase subunit β; hsp60, heat shock protein of 60 kDa; hsp70, heat shock protein of 70 kDa; MOM19, mitochondrial outer membrane protein of 19 kDa; p-, i-, m-, precursor-, intermediate-, and mature-sized forms of a protein, respectively; MOPS, 3-(*N*-morpholino)propane-sulfonic acid.

mM sucrose, 3% (w/v) BSA, 5 mM MgCl<sub>2</sub>, 80 mM KCl, 10 mM MOPS, adjusted to pH 7.2 with KOH) in a final volume of 200  $\mu$ l.

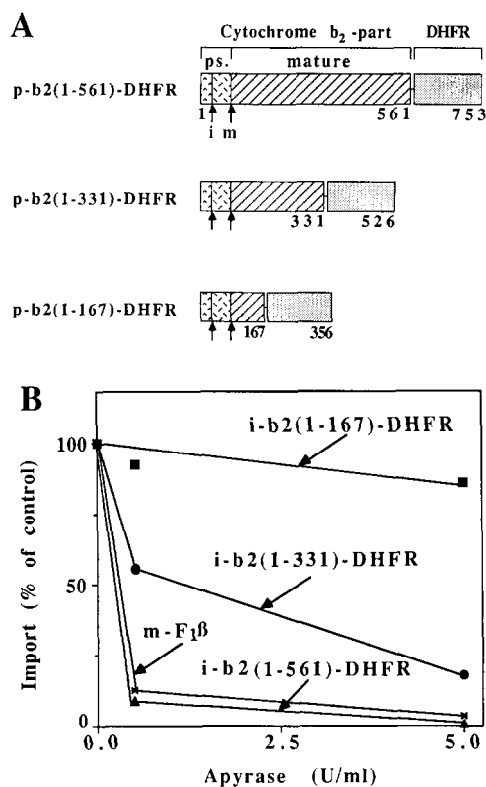
## RESULTS

**ATP Dependence of Mitochondrial Import of Cytochrome *b*<sub>2</sub>-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*<sub>2</sub> (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*<sub>2</sub> precursor polypeptide (Fig. 1A). The cell free import system, isolated *N. crassa* mitochondria and rabbit reticulocyte lysate containing the [<sup>35</sup>S]methionine-labeled precursor proteins, was depleted of ATP by preincubation with apyrase. Oligomycin was included to prevent synthesis of ATP by the F<sub>0</sub>F<sub>1</sub>-ATPase. Pretreatment with apyrase, an ATPase and ADPase from potato, had been found to inhibit mitochondrial import of many precursor proteins, e.g. F<sub>1</sub>-ATPase subunit  $\beta$  (F<sub>1</sub> $\beta$ ), and this inhibition could be reversed by readdition of ATP but not by the addition of nonhydrolyzable ATP analogues (19, 23, 27, 28). Import of the hybrid protein *b*<sub>2</sub>(1-561)-DHFR was inhibited to the same extent as import of F<sub>1</sub> $\beta$  by depletion of ATP, while inhibition of import of *b*<sub>2</sub>(1-331)-DHFR was less pronounced (Fig. 1B). Import of *b*<sub>2</sub>(1-167)-DHFR was practically not affected by the pretreatment with apyrase (Fig. 1B). It appeared that the shorter the cytochrome *b*<sub>2</sub> portion of the hybrid protein was, the less ATP was required for import.

We recently reported that unfolding of the DHFR domain of *b*<sub>2</sub>(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 Are Independent of Added ATP**—We first investigated if under the conditions used the DHFR domain of the hybrid protein *b*<sub>2</sub>(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*<sub>2</sub>(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*<sub>2</sub>(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 DHFR-containing fragment with high protease resistance (Fig. 2B).

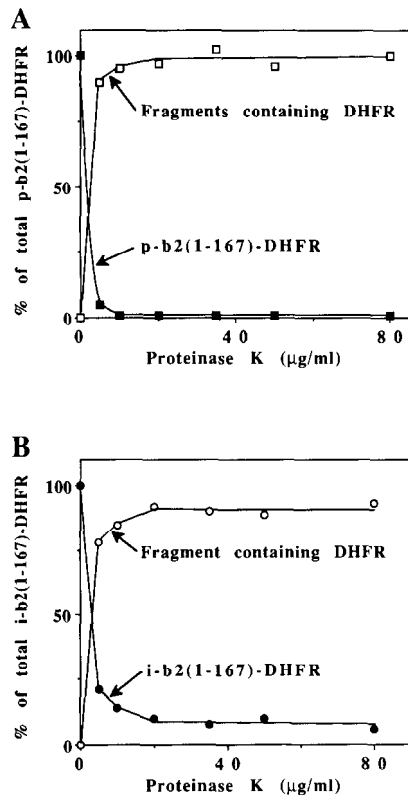
Translocation of *b*<sub>2</sub>(1-167)-DHFR across the mitochondrial membranes can be experimentally divided into two steps (13). First, the cytochrome *b*<sub>2</sub> portion is inserted into contact sites (10); the presequence is proteolytically processed by the processing peptidase in the mitochondrial 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*<sub>2</sub>(1-167)-DHFR was analyzed. Translocation of *b*<sub>2</sub>(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<sub>1</sub> $\beta$  into contact sites strongly depended on the presence of ATP (Fig. 3A) (23, 27, 30). For the second step, *b*<sub>2</sub>(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*<sub>2</sub>(1-167)-DHFR (Fig. 3B). In contrast, completion of import



**FIG. 1. Import of cytochrome *b*<sub>2</sub>-DHFR hybrid proteins into mitochondria.** A, cytochrome *b*<sub>2</sub>-DHFR hybrid proteins. The hybrid proteins were constructed as described under "Materials and Methods" and Ref. 13. Numbers indicate amino acid residues starting with the amino-terminal residue of the precursor of yeast cytochrome *b*<sub>2</sub> (46). The following linker amino acid residues were inserted between the cytochrome *b*<sub>2</sub> part and entire mouse DHFR: Arg, Gly, Ser, Gly, Ile (*b*<sub>2</sub>(1-561)-DHFR); Pro, Gly, Ile, His, Arg, Ser, Gly, Ile (*b*<sub>2</sub>(1-331)-DHFR); Gly, Ile (*b*<sub>2</sub>(1-167)-DHFR). The arrows indicate the sites of proteolytic processing of the presequence (ps.) of cytochrome *b*<sub>2</sub>; cleavage to the intermediate-sized form (*i*) probably occurs between the amino acid residues 31 and 32 (13); cleavage to the mature-sized form (*m*) occurs between amino acid residues 80 and 81 (47). In *N. crassa* mitochondria only cleavage to the intermediate-sized form is observed (13). B, inhibition of import by pretreatment with apyrase. Rabbit reticulocyte lysate containing radiolabeled precursor proteins and isolated *N. crassa* mitochondria were depleted of ATP and ADP by preincubation with apyrase (23, 41) as described under "Materials and Methods." Mitochondria and reticulocyte lysate were incubated in the presence of potassium ascorbate and *N,N,N',N'*-tetramethylphenylenediamine (to generate a mitochondrial membrane potential) and oligomycin (to block the F<sub>0</sub>F<sub>1</sub>-ATPase) under standard import conditions (see "Materials and Methods") for 15 min at 25 °C. Then treatment with proteinase K (30  $\mu$ g/ml) was performed (24). The mitochondria were reisolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Import was assessed by laser densitometry of the protease-protected intermediate-sized and mature-sized forms on the fluorographs. The import in control reactions that received heat-inactivated apyrase was set to 100% for each precursor protein. A similar dependence of import on ATP levels was found when the treatment with protease was omitted and the generation of the intermediate-sized and mature-sized forms was taken as measurement for import (see also Fig. 3A). Two independent controls confirmed that the protease-protected *i*-*b*<sub>2</sub>(1-167)-DHFR was imported also in the presence of apyrase; it was not accessible to specific antibodies added to intact mitochondria, whereas *i*-*b*<sub>2</sub>(1-167)-DHFR accumulated in contact sites was accessible to externally added antibodies (data not shown), similar to results reported previously for F<sub>1</sub> $\beta$  (30); and, it was found to be associated<sup>3</sup> with hsp60 in the mitochondrial matrix (9).

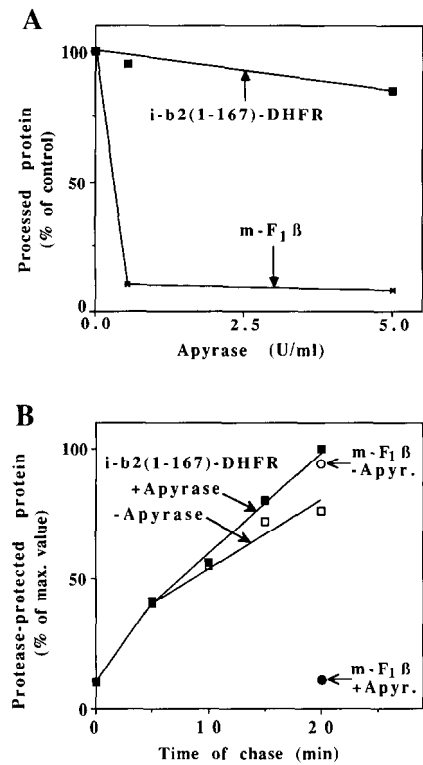
of F<sub>1</sub> $\beta$  strongly depended on the presence of ATP, i.e. was inhibited by the treatment with apyrase (Fig. 3B) (27, 31).

*The Hybrid Protein *b*<sub>2</sub>(1-167)-DHFR Uses the Import*



**FIG. 2. The DHFR domain of  $b_2(1-167)$ DHFR is in a folded conformation.** *A*,  $b_2(1-167)$ -DHFR synthesized *in vitro*. Reticulocyte lysate containing radiolabeled  $b_2(1-167)$ -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).  $p$ - $b_2(1-167)$ -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  $p$ - $b_2(1-167)$ -DHFR and the DHFR-containing fragments, the different number of methionines in precursor and fragments was taken into consideration. *B*,  $b_2(1-167)$ -DHFR accumulated in contact sites. The experiment was performed as described in the legend of Fig. 1*B* except that the pretreatment with apyrase was omitted and that the import reaction was incubated for 20 min at 8 °C. The mitochondria containing  $i$ - $b_2(1-167)$ -DHFR accumulated in contact sites were reisolated, 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  $i$ - $b_2(1-167)$ -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  $b_2(1-167)$ -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  $b_2(1-167)$ -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  $F_1\beta$  (15). IgGs directed against MOM19 were bound to mitochondria and import of  $b_2(1-167)$ -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 preimmune sera, did not inhibit. We conclude that  $b_2(1-167)$ -DHFR employs the receptor MOM19, leading to the interesting notion that binding of a precursor to and release from



**FIG. 3. ATP dependence of distinct steps in import of  $b_2(1-167)$ -DHFR.** *A*, transport into contact sites. The experiment was performed as described in the legend of Fig. 1*B* 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  $i$ - $b_2(1-167)$ -DHFR and 88% of  $m$ - $F_1\beta$  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.

MOM19 appear to be ATP-independent. The conclusion that  $b_2(1-167)$ -DHFR uses the same mitochondrial import site as ATP-dependent precursor proteins is further supported by the finding that  $b_2(1-167)$ -DHFR accumulated in contact sites inhibits import of other precursor proteins such as  $F_1\beta$ , 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  $b_2(1-167)$ -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  $F_0$ -ATPase subunit 9 ( $F_09$ ). The precursor of  $F_09$



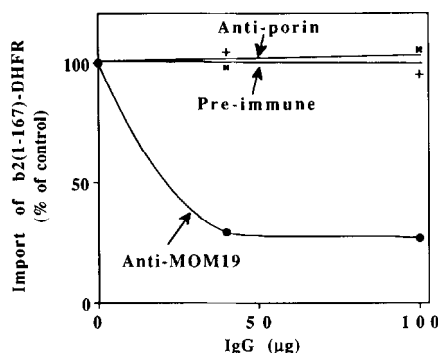


FIG. 4.  $b_2(1-167)$ -DHFR uses the import receptor MOM19. The experiment was performed as described in the legend of Fig. 1B with the following modifications. The pretreatment with apyrase and the addition of antimycin A and oligomycin were omitted; the mitochondria were preincubated with the indicated IgGs as described (15). The amount of  $i$ - $b_2(1-167)$ -DHFR imported into mitochondria that had been pretreated with trypsin (15  $\mu\text{g}/\text{ml}$ ) (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.

contains a stably folded domain (32) and was found to be efficiently imported into mitochondria at very low levels of ATP (27), indicating that targeting, unfolding, and membrane translocation of this authentic mitochondrial precursor protein may also be independent of ATP. The observation that a fusion protein between  $F_09$  and DHFR required ATP for import (27) indicates that an ATP-independent import is a property specific for the complete precursor and not just caused by the presence of DHFR in a precursor, similar to the results obtained with the longer  $b_2$ -DHFR fusion proteins (Fig. 1B). The  $F_09$ -DHFR fusion protein might, for example, possess an unfavorable conformation and therefore depend on the assistance of ATP and cytosolic cofactors for membrane translocation.

#### DISCUSSION

We describe the seemingly paradoxical situation that both tightly folded precursor proteins ((27) this study) and highly unfolded precursor proteins (8, 9) do not require ATP for import into mitochondria. Moreover, both a stably folded precursor protein (11) and an unfolded precursor protein (8, 33) were found not to depend on cytosolic cofactors for import. On the other hand, most mitochondrial precursor proteins do depend on both ATP and cytosolic cofactors including 70-kDa stress proteins (hsp70s) (1, 2, 7, 27, 34). How can this apparent discrepancy be explained? Most precursor proteins obviously do not fold to their stable mature forms in the cytosol (summarized in Ref. 6). At the same time, they are not fully unfolded (8, 27). We argue that in the absence of ATP and cofactors intra- or intermolecular interactions are locking the precursors in conformational states that cannot be resolved so to allow passage through the membranes. ATP and cytosolic cofactors seem to be required to prevent or reverse such improper folding and interactions of proteins. Artificial unfolding of precursors allows a rapid import because thereby the usually rate-limiting unfolding reaction is circumvented (8, 9). The transport-competent state of such precursors, and consequently their independence of ATP and cofactors, has only a short lifetime. This is in agreement with the expected formation of improper structures in the absence

of import into mitochondria.<sup>2</sup> Tightly and correctly folded domains such as the DHFR part most likely do not undergo unspecific interactions that might interfere with import. In support of this, we found that the ATP requirement for import of  $b_2$ -DHFR fusion proteins became higher the longer the amino-terminal portion was, *i.e.* the higher the chance of improper folding or interaction of the cytochrome  $b_2$  part was. Evidence for a role of ATP in modulating the tertiary and quaternary structures of cytosolic precursors in the absence of mitochondria is also provided by the following observations. The conformational state (as assessed by the sensitivity to added proteases) of precursor proteins synthesized in rabbit reticulocyte lysate was dependent on the levels of ATP (27). A mutant form of  $F_1\beta$  lacking an internal oligomer-forming sequence required less ATP for import than the authentic precursor that was present in an oligomeric complex in the cytosol (35).

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 proteins 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  $b_2(1-167)$ -DHFR indeed interacts with hsp60 in a

<sup>2</sup> R. Pfaller, N. Pfanner, and W. Neupert, unpublished data.

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

| 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 mitochondria                | (i) Association of precursors with cytosolic protein factors | +                 | AAC, porin, F <sub>1</sub> β, cytochromes b <sub>2</sub> and c <sub>1</sub>                                                   |
|                                                                    |                                     | or (ii) native folding of precursor domain(s)                | −                 | b <sub>2</sub> (1-167)-DHFR, F <sub>0</sub> 9                                                                                 |
|                                                                    |                                     | or (iii) artificial unfolding of precursors                  | −                 | Porin, Fe/S protein, F <sub>0</sub> 9-DHFR fusion proteins                                                                    |
| 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                                                | +                 | F <sub>1</sub> β, F <sub>0</sub> 9, Fe/S protein, cytochromes b <sub>2</sub> and c <sub>1</sub> , b <sub>2</sub> (1-167)-DHFR |
|                                                                    |                                     | or (ii) without hsp60                                        | −                 | AAC                                                                                                                           |

reaction involving ATP hydrolysis<sup>3</sup>; this step is not directly necessary for membrane translocation. Moreover, recent studies suggest that a hsp70 in the mitochondrial matrix, termed Ssc1p (44), binds to precursor proteins in transit through contact sites and thereby supports the translocation of the precursors.<sup>4</sup> Ssc1p then apparently mediates the transfer of the precursors to hsp60.<sup>4</sup> The binding of precursors to the chaperone Ssc1p in the matrix could provide the energy for membrane translocation. ATP hydrolysis might be required for release of the precursors from Ssc1p (36, 38, 39), setting the chaperone free for new rounds of import. It is unknown if ATP that could be tightly bound to Ssc1p (and would thus not be removed by the treatment with apyrase) is already needed for binding of precursors to Ssc1p.

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 sorting and assembly (45). 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.

**Acknowledgment**—We thank Christine Forster for expert technical assistance.

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