Molecular Biology of the Cell
Vol. 5, 465–474, April 1994

Protein Import into Mitochondria: the Requirement for External ATP Is Precursor-specific Whereas Intramitochondrial ATP Is Universally Needed for Translocation into the Matrix

Clemens Wachter, Gottfried Schatz, and Benjamin S. Glick

Biozentrum, CH-4056 Basel, Switzerland

Submitted December 27, 1993; Accepted February 22, 1994
Monitoring Editor: Randy W. Schekman

ATP is needed for the import of precursor proteins into mitochondria. However, the role of ATP and its site of action have been unclear. We have now investigated the ATP requirements for protein import into the mitochondrial matrix. These experiments employed an in vitro system that allowed ATP levels to be manipulated both inside and outside the mitochondrial inner membrane. Our results indicate that there are two distinct ATP requirements for mitochondrial protein import. ATP in the matrix is always needed for complete import of precursor proteins into this compartment, even when the precursors are presented to mitochondria in an unfolded conformation. In contrast, the requirement for external ATP is precursor-specific; depletion of external ATP strongly inhibits import of some precursors but has little or no effect with other precursors. A requirement for external ATP can often be overcome by denaturing the precursor with urea. We suggest that external ATP promotes the release of precursors from cytosolic chaperones, whereas matrix ATP drives protein translocation across the inner membrane.

INTRODUCTION

Mitochondria are a useful system for studying the membrane translocation and intraorganellar sorting of proteins (for reviews see Glover and Lindsay, 1992; Segui-Real *et al.*, 1992; Hannavy *et al.*, 1993). Protein import into mitochondria requires two forms of energy: an electrochemical potential across the inner membrane and ATP (Beasley *et al.*, 1992; Glick *et al.*, 1992b). Although the function of the electrochemical potential is not completely understood, it appears that the potential drives the initial insertion of a precursor into and partially across the inner membrane (Schleyer and Neupert, 1985; Martin *et al.*, 1991). A requirement for ATP was first revealed using in vitro import systems (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers *et al.*, 1987). However, the mechanism of ATP action remained obscure. Originally it was proposed that ATP hydrolysis acts outside the mitochondria to keep precursor proteins in an import-competent, unfolded conformation (Chen and Douglas, 1987; Eilers *et al.*, 1987; Pfanner *et al.*, 1987). This model was supported by the observation that the ATP requirement for import could apparently be overcome by unfolding the precursor proteins, either by prematurely arresting translation (Verner and Schatz, 1987), by introducing internal deletions (Chen and Douglas, 1988), or by acid-base treatment (Pfanner *et al.*, 1988). However, a fusion protein containing a tightly folded dihydrofolate reductase (DHFR) moiety was shown to unfold at the mitochondrial surface independently of ATP (Eilers *et al.*, 1988). Additional findings made the picture even more complex. One study concluded that a urea-denatured precursor still needed ATP for import into the matrix (Eilers *et al.*, 1988), whereas a subsequent study found no such requirement (Ostermann *et al.*, 1989). Experiments with an intermembrane space-targeted DHFR fusion protein suggested that this precursor could cross the inner membrane in the complete absence of ATP (Pfanner *et al.*, 1990). On the other hand, it was shown that import of a matrix-targeted DHFR fusion protein did require ATP and that ATP was utilized in the matrix rather than outside the mitochondria (Hwang and Schatz, 1989).

To clarify the role of ATP in mitochondrial protein import, we developed an in vitro import system in which

ATP levels could be selectively manipulated both inside and outside the mitochondrial inner membrane (Hwang and Schatz, 1989; Wachter *et al.*, 1992; Glick *et al.*, 1993). This system allowed us to investigate the functions of ATP in the import and sorting of precursors targeted to the different mitochondrial subcompartments. One important conclusion from this work was that in the absence of matrix ATP, many precursors can still be imported across the outer membrane (Hwang *et al.*, 1991). Indeed, several precursors targeted to the intermembrane space or inner membrane follow import pathways that are independent of matrix ATP (Glick *et al.*, 1992a, 1993; Lill *et al.*, 1992; Wachter *et al.*, 1992).

In the present study we have tested the effects of ATP depletion on the import of a variety of matrix-targeted precursors. Our findings help to explain the previous discrepancies. In addition, they demonstrate that ATP has two distinct functions in mitochondrial protein import.

MATERIALS AND METHODS

General Methods

Most procedures have been described elsewhere (Glick, 1991; Glick *et al.*, 1992a; Wachter *et al.*, 1992), including preparation of Nycodenz-purified yeast mitochondria and measurement of mitochondrial protein concentration, osmotic shock treatment to generate mitoplasts, proteinase K treatment (50–100 μg/ml protease for 20–30 min on ice, followed by the addition of phenylmethanesulfonyl fluoride to 1 mM), the use of protease-sensitive markers for the matrix (α-ketoglutarate dehydrogenase) and intermembrane space (endogenous cytochrome b_2), precipitation with trichloroacetic acid, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, laser densitometry, and immunoblotting with rabbit antisera and ^{125}I-protein A. Synthesis of ^{35}S-labeled precursor proteins was performed in a reticulocyte lysate system (Hurt *et al.*, 1984) containing 5 mM creatine phosphate and 10 mM dithiothreitol (DTT). The precursor proteins used were all from *Saccharomyces cerevisiae*.

Import Reactions

Native and urea-denatured precursors were diluted 20-fold into import buffer, which contained 0.6 M sorbitol (deionized), 50 mM K^+ *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), 50 mM KCl, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 2.5 mM EDTA, 5 mM methionine, 1 mg/ml fatty acid-free bovine serum albumin, and 10 mM glycerol. Mitochondria were present at a final concentration of 0.25 mg/ml protein or 1 mg/ml for import of urea-denatured precursors. Import times for the different precursors were in the linear range: 10 min at 25°C for $F_1\beta$, 2 min at 25°C for MPPα, 0.3 min at 25°C for urea-denatured MPPα, 5 min at 25°C for the Fe/S protein, 1 min at 25°C for the urea-denatured Fe/S protein, 5 min at 20°C for ADHIII, 1 min at 20°C for urea-denatured ADHIII, 7.5 min at 15°C for CoxIV, 2 min at 8°C for hsp60, 5 min at 8°C for $F_1\beta$-Δ(94-381), and 5 min at 12°C for $F_1\beta$-Δ(123-381). At higher temperatures the $F_1\beta$ deletion mutants are rapidly degraded upon import into the matrix (Hines, unpublished data). For import of urea-denatured precursors, proteins in the reticulocyte lysate were precipitated with 67% ammonium sulfate for 20 min on ice, followed by centrifugation for 10 min at 12 000 × *g*; the pellet was resuspended in four times the original volume of 8 M urea, 20 mM tris(hydroxymethyl)-aminomethane-HCl (pH 7.0), 10 mM DTT. Import reactions were terminated by adding carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) to 25 μM and transferring the mixtures to ice. After import, mitochondria were centrifuged 3 min at 12 000 × *g* and resuspended in import buffer containing 25 μM FCCP. Mitochondria or mitoplasts were then treated with proteinase K. Finally, the organelles were reisolated once again and precipitated with trichloroacetic acid. To confirm that the outer membrane had remained sealed in the intact mitochondria but had been selectively ruptured upon mitoplast formation, we subjected a portion of each sample to SDS-PAGE followed by immunoblotting for cytochrome b_2 and α-ketoglutarate dehydrogenase (see Glick *et al.*, 1992a).

Spin-Desalting of the Reticulocyte Lysate After Precursor Synthesis

G-25 Sephadex (fine grade; Sigma, St. Louis, MO) columns (1 ml bed volume) were equilibrated in 100 mM KCl, 20 mM K^+HEPES (pH 7.4), 5 mM $MgCl_2$, and 10 mM DTT and were prespun for 2 min at 1000 × *g*. Postribosomal translation mixture (75-μl aliquots, pretreated with hexokinase/2-deoxyglucose as described below) was then spun for 2 min at 1000 × *g* through the columns. This treatment removed small molecules, including 2-deoxyglucose and free nucleotides.

Manipulation of ATP Levels

We improved and simplified our previous methods for ATP depletion (Wachter *et al.*, 1992; Glick *et al.*, 1993). After synthesis of the ^{35}S-labeled precursor protein, the reticulocyte lysate was placed ≥5 min on ice, and then ATP was depleted by incubating 5 min at 30°C with 50 U/ml yeast hexokinase plus 15 mM 2-deoxyglucose. Ribosomes were removed by centrifugation for 10 min at 150 000 × *g*, and the precursor was diluted into an import mixture containing mitochondria that had been pretreated as described below. Efrapeptin and oligomycin were used to inhibit the mitochondrial ATP synthase. Carboxyatractyloside (CAT) was added to inhibit the adenine nucleotide translocator and thereby block passage of ATP and ADP across the inner membrane. α-ketoglutarate was used to regenerate matrix ATP by substrate level phosphorylation (Glick *et al.*, 1993). Glycerokinase from *Escherichia coli* cleaves ATP to ADP in the presence of glycerol; this enzyme is highly specific for ATP (Thorner and Paulus, 1973). For all four ATP conditions, mitochondria were preincubated 5 min at 30°C in import buffer containing 2 μg/ml efrapeptin, 5 μg/ml oligomycin, and 25 μg/ml CAT. This procedure efficiently depletes free ATP in the mitochondrial matrix (Glick *et al.*, 1993). NADH was then added to 2 mM to generate an electrochemical potential across the inner membrane (von Jagow and Klingenberg, 1970). The mitochondrial suspension was divided into four aliquots corresponding to the different ATP conditions. These aliquots were treated as follows: ATP in+out: ATP was present both inside and outside the inner membrane. α-ketoglutarate was added to 5 mM to generate matrix ATP, and after 3 min at the import temperature, 0.5 mM ATP and an ATP-regenerating system (5 mM creatine phosphate, 100 μg/ml rabbit muscle creatine kinase) were added, followed by the hexokinase-treated precursor. ATP in: ATP was depleted outside the inner membrane but was present in the matrix. α-ketoglutarate and glycerokinase were added to 5 mM and 10 U/ml, respectively, followed by incubation for 3 min at the import temperature before addition of the hexokinase-treated precursor. ATP out: ATP was depleted in the matrix but was present outside the inner membrane. After incubating 3 min at the import temperature, 0.5 mM ATP plus an ATP-regenerating system were added, followed by the hexokinase-treated precursor. No ATP: ATP was depleted on both sides of the inner membrane. Glycerokinase was added to 10 U/ml, and after 3 min at the import temperature, the hexokinase-treated precursor was added.

Miscellaneous

CAT was purchased from Fluka (Buchs, Switzerland), and efrapeptin was a gift of the Eli Lilly (Indianapolis, IN); other reagents were purchased from Sigma (St. Louis, MO). The plasmids encoding $F_1\beta$-Δ(94-381) and $F_1\beta$-Δ(123-381) were kindly provided by Michael Douglas,

University of North Carolina, Chapel Hill, and the plasmid encoding the Fe/S protein was kindly provided by Diana Beattie, West Virginia University School of Medicine, Morgantown.

RESULTS

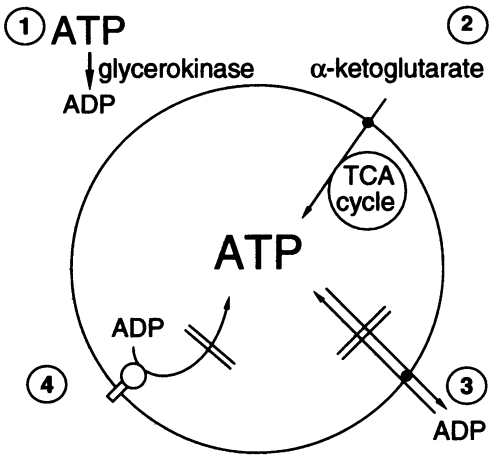
An Experimental System for Studying the Role of ATP in Mitochondrial Protein Import

We developed an improved experimental system that allowed for a comparison of protein import rates under four different conditions: ATP both inside and outside the inner membrane, ATP only in the matrix, ATP only outside the inner membrane, or no ATP in either location (Figure 1). In all cases, transport of ATP and ADP across the inner membrane was prevented by adding CAT. In addition, the mitochondrial ATP synthase was inhibited by using a combination of oligomycin and efrapeptin; this treatment allows endogenous ATPases to hydrolyze any ATP that may be present in the matrix (Glick *et al.*, 1993). Where indicated, matrix ATP levels were restored by adding α-ketoglutarate, which leads to ATP formation by substrate-level phosphorylation (Glick *et al.*, 1993). Precursor proteins were synthesized in a reticulocyte lysate, and this mixture was depleted of ATP with hexokinase/2-deoxyglucose. ATP levels outside the mitochondria were then either restored by adding ATP or kept low by adding glycerokinase/glycerol.

In the past, various other combinations of inhibitors and pretreatments have been used to selectively deplete either matrix ATP or external ATP (e.g., Chen and Douglas, 1987; Hwang and Schatz, 1989; Cyr *et al.*, 1993). We consider the procedure described above to be superior for two reasons. First, in all cases the system is initially depleted of external ATP. The inhibitory effect of this depletion is determined by comparison with a sample in which external ATP levels have been restored; thus the inhibition that is measured is reversible and presumably specific (Glick *et al.*, 1993). Second, earlier methods relied on the mitochondrial ATP synthase to generate matrix ATP, so that oligomycin was added only to those samples in which matrix ATP was being depleted. However, oligomycin has a nonspecific inhibitory effect on the import of certain precursors, such as cytochrome c_1 (Wachter *et al.*, 1992; Glick *et al.*, 1993). We have not observed any nonspecific effects when matrix ATP is generated by adding α-ketoglutarate. Therefore the procedure outlined in Figure 1 allows us for the first time to make simultaneous, quantitative comparisons between all four different ATP conditions.

Complete Import of Matrix-targeted Precursors Always Requires Matrix ATP Whereas the Requirement for External ATP Is Precursor-specific

Several authentic matrix-targeted precursors were incubated with mitochondria under different ATP conditions (Figures 2 and 3). Import times were chosen to



	ATP			
	In + out	In	out	none
oligomycin/efrapeptin	+	+	+	+
carboxyatractyloside	+	+	+	+
α-ketoglutarate	+	+	-	-
ATP	+	-	+	-
glycerokinase/glycerol	-	+	-	+

Figure 1. Experimental system for studying the ATP requirements for mitochondrial protein import. Top: Schematic drawing of the experimental system. Only the inner mitochondrial membrane is represented because the outer membrane is permeable to small molecules. (1) ATP is either added to the incubation medium or is converted to ADP by an enzymatic trap such as glycerokinase/glycerol. (2) To generate matrix ATP, α-ketoglutarate is added. This metabolite is transported into the matrix by a specific carrier and then produces ATP by substrate-level phosphorylation in the tricarboxylic acid (TCA) cycle. (3) CAT is added to inhibit the adenine nucleotide translocator and prevent passage of ATP and ADP across the inner membrane. (4) The mitochondrial ATP synthase is blocked by adding a combination of oligomycin and efrapeptin. Bottom: Summary of the procedures used to generate the four different ATP conditions.

be in the linear range for each precursor. Translocation across the outer membrane was assayed by treating the intact mitochondria with protease after the incubation; translocation across the inner membrane was assayed by protease treatment of mitoplasts, in which the outer membrane had been ruptured by swelling in hypotonic medium.

From the results of these experiments, the precursors could be classified into two groups. The first group (Figure 2) consisted of the β-subunit of the F_1-ATPase ($F_1\beta$), the α-subunit of the matrix processing peptidase (MPPα), the iron-sulfur protein of cytochrome c reductase (Fe/S), and mitochondrial alcohol dehydrogenase (ADHIII). When ATP was present only in the matrix (ATP in), import of these precursors was reduced ≥70% relative to the condition in which ATP was present on both sides of the inner membrane (ATP in+out). Depletion of ATP on both sides of the inner membrane

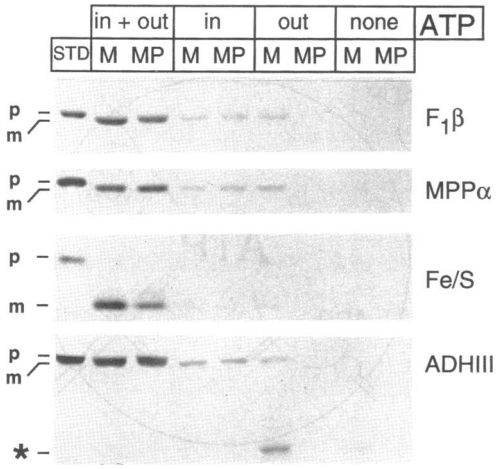


Figure 2. Complete import of several matrix-targeted precursors requires both external ATP and matrix ATP. Radiolabeled precursor proteins and mitochondria were pretreated as described in MATERIALS AND METHODS to create conditions in which ATP was present on both sides of the inner membrane (in+out), only in the matrix (in), only outside the inner membrane (out), or in neither location (none). After import, the mitochondria (M) were either treated with proteinase K or converted to mitoplasts (MP) in the presence of proteinase K. All samples were then precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. $F_1\beta$, β-subunit of the F_1-ATPase; MPPα, α-subunit of the presequence processing protease (formerly known as MAS2p); Fe/S, iron-sulfur protein of cytochrome c reductase; ADHIII, mitochondrial alcohol dehydrogenase; STD, 10% of the amount of precursor mixture initially added to each sample; p and m, precursor and mature forms of the radiolabeled precursor proteins. With ADHIII, a protease-protected fragment of ~30 kDa (*) was generated upon import into mitochondria depleted of matrix ATP.

(ATP none) inhibited import completely. In the presence of only external ATP (ATP out), there was no detectable import across the inner membrane; however, the precursors were still translocated across the outer membrane, albeit with reduced efficiency. Similar translocation intermediates located between the two membranes have been described previously (Hwang *et al.*, 1991; Rassow and Pfanner, 1991).

ADHIII formed an additional translocation intermediate when ATP was present only outside the mitochondria. Under these conditions some of the ADHIII molecules were arrested during translocation across the outer membrane, so that protease treatment of mitochondria generated a protected fragment of ~30 kDa (Figure 2, asterisk). The arrested ADHIII molecules could be chased across the outer membrane by prolonged incubation at 30°C, or they could be chased completely into the matrix by restoration of matrix ATP levels. Accumulation of this intermediate was probably caused by a folded structure within the ADHIII precursor.

The second group of precursors (Figure 3) included subunit IV of cytochrome oxidase (CoxIV) and mitochondrial hsp60. Depletion of external ATP had no significant effect on the import of these precursors. Depletion of matrix ATP completely blocked import of both CoxIV and hsp60, but when the incubations were performed at 25–30°C rather than 8°C, hsp60 was translocated across the outer membrane of ATP-depleted mitochondria (Hwang *et al.*, 1991; Wachter *et al.*, 1992). Thus the precursors shown in Figure 3 are imported independently of external ATP, but in other respects they resemble the precursors shown in Figure 2.

It was reported that two mutant forms of $F_1\beta$ with large internal deletions, $F_1\beta$-Δ(94-381) and $F_1\beta$-Δ(123-381), could be imported into mitochondria that had been treated with apyrase (Chen and Douglas, 1988). This result was interpreted to mean that import of these mutant proteins was independent of ATP. However, the ATP synthase had not been inhibited in the earlier experiments, so ATP was presumably still present in the matrix. We therefore reinvestigated the import of these two precursors using our experimental system. Import of $F_1\beta$-Δ(94-381) (Figure 3) and $F_1\beta$-Δ(123-381) (see Cyr *et al.*, 1993) was completely blocked in the absence of matrix ATP, whereas depletion of external ATP had no effect. The previously published results can therefore be explained by assuming that ATP was present in the matrix but not outside the mitochondria. Interestingly, the external ATP requirement for import of $F_1\beta$ (Figure 2) was abolished by making internal deletions in this precursor.

We conclude that the complete translocation of precursor proteins across the inner membrane invariably requires ATP in the matrix. However, external ATP is required only for the import of some mitochondrial precursors.

Denaturation of Precursor Proteins Can Overcome an External ATP Requirement for Import

Precursors that require external ATP for import are presumably associated with cytosolic chaperones (see DIS-

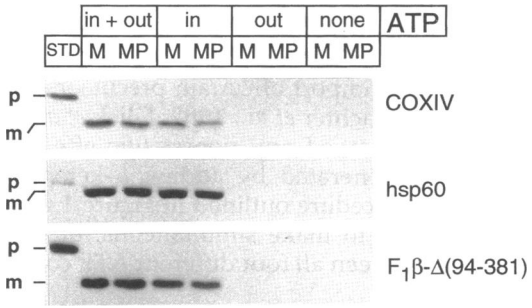


Figure 3. Some matrix-targeted precursors require matrix ATP but not external ATP for translocation across the inner membrane. Import reactions and subsequent treatments were as in Figure 2. CoxIV, subunit IV of cytochrome oxidase; hsp60, 60-kDa mitochondrial chaperonin; $F_1\beta$-Δ(94-381), mutant form of $F_1\beta$ with an internal deletion comprising amino acids 94–381 of the precursor protein; STD, p, and m, as in Figure 2.

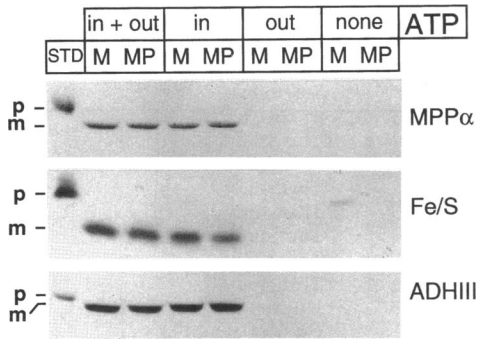


Figure 4. Urea denaturation can overcome an external ATP requirement for import but does not overcome the matrix ATP requirement for complete translocation across the inner membrane. Precursor proteins were denatured in 8 M urea and diluted into import buffer containing mitochondria that had been pretreated as in Figure 2. Further processing of the samples was as in Figure 2. STD, precursor standards (10% for MPPα and the Fe/S protein, 5% for ADHIII); p and m, as in Figure 2.

CUSSION). High concentrations of urea should disrupt such associations, potentially allowing for import in the absence of external ATP. This possibility has not been tested in previous studies, which examined the effects of urea denaturation on folded precursors that are imported independently of external ATP (Eilers *et al.*, 1988; Gambill *et al.*, 1993; Glick *et al.*, 1993). We therefore analyzed the import of the urea-denatured precursors of ADHIII, MPPα, and the Fe/S protein. All three precursors could be imported in the absence of external ATP if they were presented to mitochondria in a urea-denatured form (Figure 4). However, urea denaturation did not overcome the matrix ATP requirement for translocation across the inner membrane (Figure 4), even with a precursor as small as CoxIV (17 kDa). Thus denaturation of a matrix-targeted precursor can eliminate a requirement for external ATP but does not allow complete import across the inner membrane in the absence of matrix ATP.

The External ATP-dependent Step of Import Shows a Strict Specificity for Hydrolyzable ATP

Several other nucleoside triphosphates were previously found to substitute for ATP in promoting import of mitochondrial precursors (Chen and Douglas, 1987; Eilers *et al.*, 1987; Pfanner *et al.*, 1987). However, conversion of these nucleotides into ATP was not excluded. It was shown that GTP needs to be converted into ATP to fulfill the matrix ATP requirement for import, presumably because only ATP is transported efficiently across the inner membrane (Hwang and Schatz, 1989). To determine the specificity of the external nucleotide requirement for import, we first spun a precursor mixture through a desalting column to remove small molecules, then added various nucleotides and tested their ability to restore import. This experiment was performed with the precursors of MPPα and the adenine nucleotide translocator, both of which require external ATP for efficient import (see Figure 2 and Wachter *et al.*, 1992). Neither ADP, GTP, CTP, nor nonhydrolyzable ATP analogues could substitute for external ATP in stimulating import of MPPα (Figure 5) or the adenine nucleotide translocator. With both precursors, maximal import rates were achieved with 100–300 μM ATP. These concentrations probably overestimate the actual K_m for the ATP-dependent step of import because there are many ATPases present in the reticulocyte lysate. Nevertheless, it seems clear that hydrolysis of external ATP is needed to confer import competence to certain precursor proteins.

DISCUSSION

The transport of proteins across biological membranes requires energy. In this study we have concentrated on the role of ATP in the import of proteins into the mitochondrial matrix. Our results, together with additional data from other studies, are summarized in Table 1. They allow the following conclusions.

1) There are two distinct functions of ATP in mitochondrial protein import. One function involves external ATP (i.e., ATP outside the inner membrane), and the other involves matrix ATP.

2) In our in vitro system, the external ATP requirement for import is restricted to a subset of mitochondrial precursors.

3) External ATP cannot be replaced by other nucleotides.

4) The requirement for external ATP can be bypassed by altering the conformation of a precursor protein.

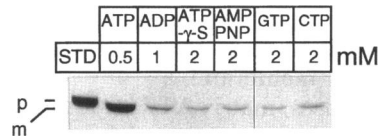


Figure 5. External ATP cannot be replaced by other nucleotides or by nonhydrolyzable ATP analogues. Reticulocyte lysate containing the radiolabeled MPPα precursor was depleted of ATP and spin-desalted on a G-25 column as described in MATERIALS AND METHODS. Mitochondria were pretreated as described for ATP in+out conditions, except that ATP and an ATP-regenerating system were omitted. The mitochondria were then divided into six equal samples, which received the following supplements: 1) 500 μM ATP, 2) 1 mM ADP plus 10 U/ml *E. coli* glycerokinase, 3) 2 mM adenosine-5′-O-(3-thiotriphosphate) (ATP-γ-S) plus 10 U/ml glycerokinase and 10 U/ml myokinase, 4) 2 mM adenylyl-imidodiphosphate (AMP-PNP) plus 10 U/ml glycerokinase and 10 U/ml myokinase, 5) 2 mM GTP plus 10 U/ml glycerokinase, 6) 2 mM CTP plus 10 U/ml glycerokinase. After 3 min at 25°C, the spin-desalted precursor was added, and the incubation was continued for 2 min at 25°C before addition of FCCP. All samples were then treated with proteinase K, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE and fluorography. STD, p, and m, as in Figure 2.

Table 1. ATP requirements for import of authentic and artificial mitochondrial precursors

Precursor	Destination	Matrix ATP	External ATP	References
$F_1\beta$	Matrix	+	+	This study
MPPα[a]	Matrix	+	+	This study
Fe/S protein[a]	Matrix	+	+	This study
ADHIII[a]	Matrix	+	+	This study
hsp60	Matrix	+	−	This study
CoxIV	Matrix	+	−	This study
$F_1\beta$-Δ (94-381)	Matrix	+	−	This study
CoxIV-DHFR[b]	Matrix	+	−	Hwang and Schatz, 1989
Cytochrome b_2[c]	IMS	+	−	Glick et al., 1993
CoxVa[d]	IM	+	−	Miller and Cumsky, 1991; Cyr et al., 1993
Cytochrome c_1	IMS	−	+	Wachter et al., 1992
Adenine nucleotide translocator	IM	−	+	Wachter et al., 1992
Porin[e]	OM	−	+	Hwang and Schatz, 1989
b_2(1-167)-DHFR	IMS	−	−	Glick et al., 1992a, 1993
c_1(1-64)-DHFR	IMS	−	−	Glick et al., 1992a
Cytochrome *c* heme lyase	IMS	−	−	Lill et al., 1992

OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

[a] The requirement for external ATP can be overcome by urea denaturation (this study).

[b] Import of other matrix-targeted DHFR fusion proteins also requires only matrix ATP (Wachter et al., 1992; Wachter, unpublished data).

[c] Like authentic cytochrome b_2, fusion protein derivatives of cytochrome b_2 containing the intact heme-binding domain require only matrix ATP for import (Glick et al., 1993; Voos et al., 1993).

[d] Although CoxVa has not been tested in our experimental system, the available evidence suggests that it requires only matrix ATP for import.

[e] The requirement for external ATP can be overcome by acid-base treatment of the precursor (Pfanner et al., 1988).

Such alterations have been achieved by urea denaturation (ADHIII, Fe/S protein, MPPα), by acid-base treatment (porin), and by introducing internal deletions ($F_1\beta$). Although only a few precursors have been tested, it is likely that conformational alterations would allow most or all precursors to be imported in the absence of external ATP.

5) Complete translocation across the inner membrane always requires ATP in the matrix, even if the precursor has been denatured with urea. If the matrix is depleted of ATP, many precursors can still cross the outer membrane, but they undergo only a partial translocation across the inner membrane.

6) In some cases, matrix ATP is required even if only a portion of the precursor is translocated across the inner membrane. Examples are cytochrome b_2 and CoxVa, both of which appear to contain stop-transfer sequences specific for the inner membrane (Glick *et al.*, 1992a, 1993; Cyr *et al.*, 1993; Miller and Cumsky, 1993).

7) Matrix ATP exerts a pulling force that can drive the unfolding of a precursor protein. This phenomenon has been documented most clearly in the case of cytochrome b_2 (Glick *et al.*, 1993; Voos *et al.*, 1993).

Our data suggest that the previous contradictory observations can be attributed to four experimental limitations. First, there was often no distinction made between ATP pools outside and inside the mitochondria. Second, some protocols for depleting ATP were only partially effective because the ATP synthase was not inhibited (e.g., Chen and Douglas, 1988; Ostermann *et al.*, 1989). Third, it was assumed that matrix-targeted precursors that had been imported into ATP-depleted mitochondria were present in the matrix (e.g., Verner and Schatz, 1987), whereas it now seems likely that these precursors had actually been arrested during translocation across the inner membrane (Hwang *et al.*, 1991). Finally, intermediate forms of intermembrane space-targeted precursors were initially thought to be located in the matrix of ATP-depleted mitochondria (Pfanner *et al.*, 1990), but these intermediates were later shown to be anchored to the outer face of the inner membrane (Glick *et al.*, 1992a).

Although in vitro experiments have led to a consistent picture of the role of ATP in mitochondrial protein import, these experiments are based on heterologous import systems, and they do not address the possibility that some precursors may be imported cotranslationally in vivo (Verner, 1993). Nevertheless, mitochondrial protein import in vivo probably involves the same kinds of ATP-dependent reactions that have been characterized in vitro. The function of ATP in mitochondrial protein import appears to be linked to the action of molecular chaperone proteins (Gething and Sambrook, 1992; Craig *et al.*, 1993). Cytosolic chaperones that have been implicated in the import process include hsp70 proteins (Deshaies *et al.*, 1988; Murakami *et al.*, 1988; Smith and Yaffe, 1991); the dnaJ homologue YDJ1p/Mas5p, which apparently acts together with hsp70 (Atencio and Yaffe, 1992; Caplan *et al.*, 1992; Cyr *et al.*, 1992); presequence-binding factor (PBF), which is

not an ATPase (Murakami *et al.*, 1992); and the ATP-dependent mitochondrial import stimulation factor (MSF) (Hachiya *et al.*, 1993). Proteins such as PBF and MSF may function in vivo to increase the fidelity of targeting to mitochondria (Lithgow *et al.*, 1993). Chaperones of the hsp70 and dnaJ families apparently bind to precursors during or soon after translation (Figure 6) (Beckman *et al.*, 1990; Nelson *et al.*, 1992; Hendrick *et al.*, 1993). It was originally proposed that the major function of these chaperones was to inhibit the premature folding of precursors (Deshaies *et al.*, 1988; Rothman, 1989). Such an antifolding activity may indeed be important for the import of some precursors, particularly those that can potentially form oligomers outside the mitochondria (Mattingly *et al.*, 1993). However, tight folding of precursor proteins does not necessarily prevent import, apparently because the mitochondrial translocation machinery can perform an unfoldase function (Gambill *et al.*, 1993; Glick *et al.*, 1993). It now seems likely that the primary function of cytosolic chaperones during protein translocation is to prevent the irreversible aggregation of precursors (Ellis and van der Vies, 1991). ATP would thus be needed to dissociate the precursor-chaperone complex so that the precursor could engage the import apparatus (Figure 6, pathway 1) (Chirico, 1992).

Some precursors can be imported in the absence of external ATP. Presumably these precursors are not prone to aggregation and are therefore released from the chaperones after translation. Precursors of this type can be classified into two groups. One group of precursors fold outside the mitochondria and are then unfolded during import (Figure 6, pathway 3). Examples are cytochrome b_2 (Glick *et al.*, 1993), fusion proteins containing a DHFR moiety (Eilers *et al.*, 1988; Hwang and Schatz, 1989; Pfanner *et al.*, 1990), and a fusion protein in which a matrix-targeting signal is joined to chloramphenicol acetyltransferase (Skerjanc *et al.*, 1990). A second group of precursors remain loosely folded outside the mitochondria and yet can be imported in the absence of external ATP (Figure 6, pathway 2). Likely examples include cytochrome c heme lyase, CoxIV, CoxVa, and mutant $F_1\beta$ proteins with internal deletions (see Table 1). Although it has not been directly shown that these mitochondrial precursors are loosely folded, detailed studies of chloroplast ferredoxin have demonstrated that this precursor has little secondary structure and yet is imported independently of cytosolic chaperones (Pilon *et al.*, 1992).

The model shown in Figure 6 is still speculative because only limited information is available about the state of precursor proteins before import. However, the existing data are consistent with this model. The $F_1\beta$ precursor, which requires external ATP for import, seems to interact with the cytosolic hsp70 system (Deshaies *et al.*, 1988; Smith and Yaffe, 1991; Atencio and Yaffe, 1992; Caplan *et al.*, 1992). In contrast, DHFR fusion proteins and cytochrome b_2, which are imported independently of external ATP, fold after synthesis in a reticulocyte lysate and thus apparently do not remain bound to cytosolic chaperones.

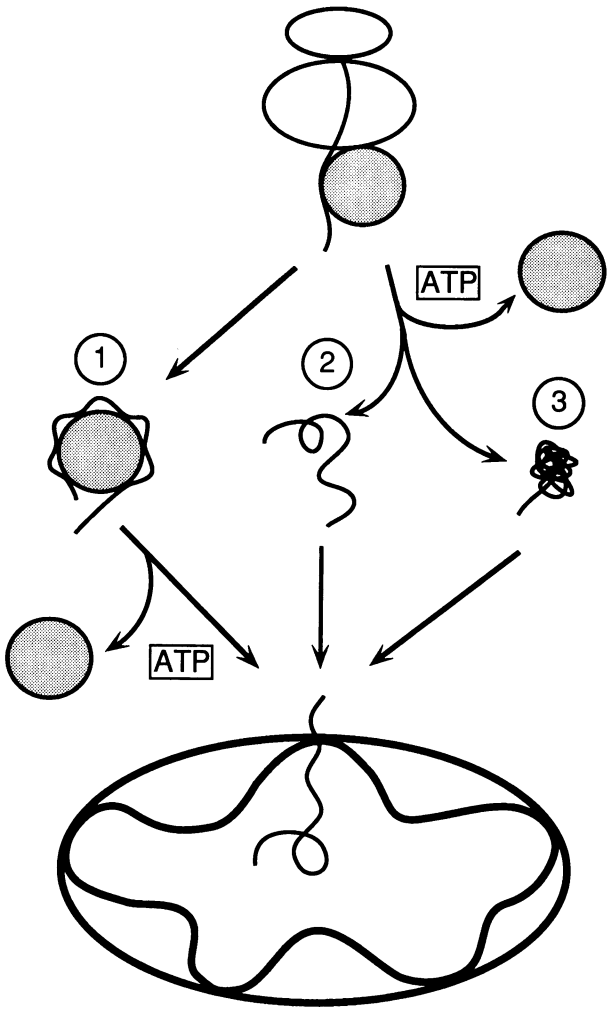


Figure 6. Proposed pathways for external ATP-dependent and external ATP-independent protein import into mitochondria. Cytosolic chaperones (◉) bind to nascent precursor chains during translation on free ribosomes. Precursors can then follow one of three pathways. 1) Cytosolic chaperones remain bound to the precursors until import occurs. Release from the chaperones requires ATP. 2) Some precursors are released rapidly from the chaperones and remain loosely folded until they engage the translocation machinery. Import is independent of external ATP. 3) Some precursors are released rapidly from the chaperones and then undergo partial or complete folding. These precursors are unfolded again during import, which is independent of external ATP. See text for details.

Destabilizing the structure of a precursor protein often accelerates import, probably by enhancing the initial interaction of the precursor with the translocation machinery (Eilers *et al.*, 1988; Vestweber and Schatz, 1988). In addition, altering the conformation of a precursor can change the energetics of import in two ways. First,

with precursors that require external ATP for import, urea denaturation circumvents this requirement, presumably by disrupting an association of the precursor with cytosolic chaperones. Other treatments that alter precursor conformation can have similar effects (Chen and Douglas, 1988; Pfanner *et al.*, 1988). Second, the initial translocation of many precursors across the outer membrane is stimulated by matrix ATP, and treatments that destabilize protein conformation can sometimes mimic this stimulatory effect (Gambill *et al.*, 1993). For example, import of cytochrome b_2 to the intermembrane space requires matrix ATP, but disrupting the folded structure of this protein allows import in the complete absence of ATP (Glick *et al.*, 1993; Voos *et al.*, 1993). Thus the pulling force generated by matrix ATP promotes unfolding of some precursors, and this function of matrix ATP can be bypassed by denaturing the precursors.

It is not known what force drives protein import across the outer membrane when the matrix is depleted of ATP. The electrochemical potential across the inner membrane is needed only for early stages of the import reaction (Schleyer and Neupert, 1985; Cyr *et al.*, 1993; Glick *et al.*, 1993). The model shown in Figure 6 predicts that external ATP would not provide energy for translocation; indeed, many precursors can reach the intermembrane space in the complete absence of ATP (Hwang *et al.*, 1991; Glick *et al.*, 1992a). One possibility is that after a precursor inserts into the inner membrane, the import channels in the two mitochondrial membranes separate from one another, thereby pulling the precursor across the outer membrane (Glick *et al.*, 1991). A different mechanism would operate for cytochrome c heme lyase, which can pass directly through the outer membrane translocation channel (Mayer *et al.*, 1993).

Matrix ATP most likely exerts its action through the matrix-localized mitochondrial hsp70 protein (mhsp70). This chaperone binds to incoming precursors and is needed for their complete translocation across the inner membrane (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991; Gambill *et al.*, 1993; Schwarz *et al.*, 1993; Voos *et al.*, 1993). It is thought that mhsp70 functions as an ATP-dependent import motor that pulls proteins into the matrix (Figure 7, left). In the case of cytochrome b_2, mhsp70 also uses ATP to exert a pulling force, which results in partial translocation of the presequence across the inner membrane and concomitant unfolding of the precursor (Figure 7, right) (Glick *et al.*, 1993; Voos *et al.*, 1993). The model of mhsp70 as a translocation ATPase agrees with most of the experimental data. One observation that remains unexplained is that neither cytochrome c_1 nor the adenine nucleotide translocator needs matrix ATP for import (Wachter *et al.*, 1992), yet import of both proteins was blocked by thermal inactivation of a temperature-sensitive mhsp70 protein (Ostermann *et al.*, 1990).

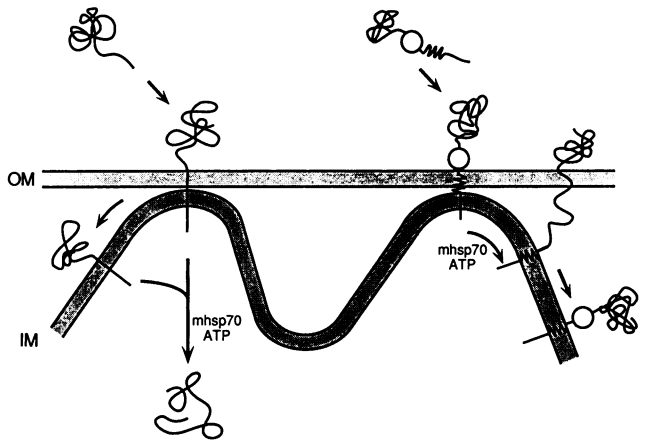


Figure 7. Proposed functions of matrix ATP in mitochondrial protein import. Left: With matrix-targeted precursors, mhsp70 uses matrix ATP to drive translocation across the inner membrane. In the absence of matrix ATP, these precursors can often cross the outer membrane to yield translocation intermediates facing the intermembrane space. Addition of ATP then allows completion of import into the matrix. Right: In the case of cytochrome b_2, mhsp70 uses matrix ATP to exert a pulling force on the presequence; this force causes the heme-binding domain in the precursor to unfold and to cross the outer membrane. The stop-transfer signal in the cytochrome b_2 presequence prevents further translocation across the inner membrane. The mechanism of ATP-dependent mhsp70 function is presumably the same for the complete import of matrix-targeted precursors and for the partial translocation of the cytochrome b_2 presequence across the inner membrane. See text for details. OM, outer membrane; IM, inner membrane.

It has been proposed that mhsp70 drives protein import by binding to precursor chains as they oscillate within the translocation channel because of Brownian motion (Neupert *et al.*, 1990; Simon *et al.*, 1992). mhsp70 binding would prevent the reverse oscillations and thereby cause unidirectional translocation into the matrix. In the original version of this Brownian ratchet model, ATP would function only to recycle mhsp70 for multiple rounds of translocation. However, matrix ATP is needed even for a single round of import, presumably because the initial association of mhsp70 with a precursor requires ATP (Manning-Krieg *et al.*, 1991; Gambill *et al.*, 1993). An updated Brownian ratchet model (Neupert and Pfanner, 1993) provides a plausible mechanism for the import of unfolded proteins. However, the finding that precursors can fold outside the mitochondria and that mhsp70 and matrix ATP drive their unfolding has lessened the appeal of this model. It may be that mhsp70 plays a more active role in protein translocation across the inner membrane. Resolution of this question will require a better understanding of the ATP-dependent reaction cycle of mhsp70.

ACKNOWLEDGMENTS

Thanks to Margrit Jäggi, Verena Grieder, and Liselotte Müller for the artwork and to the members of the Schatz lab for critical reading of

the manuscript. G.S. was supported by grant 3-26189.89 from the Swiss National Science Foundation.

REFERENCES

Atencio, D.P., and Yaffe, M.P. (1992). MAS5, a yeast homolog of DnaJ involved in mitochondrial protein import. Mol. Cell. Biol. *12*, 283–291.

Beasley, E.M., Wachter, C., and Schatz, G. (1992). Putting energy into mitochondrial protein import. Curr. Opin. Cell Biol. *4*, 646–651.

Beckman, R.P., Mizzen, L.A., and Welch, W.J. (1990). Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science *248*, 850–854.

Caplan, A.J., Cyr, D.M., and Douglas, M.G. (1992). YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell *71*, 1143–1155.

Chen, W.-J., and Douglas, M.G. (1987). Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix. Cell *49*, 651–658.

Chen, W.-J., and Douglas, M.G. (1988). An F_1-ATPase β-subunit precursor lacking an internal tetramer-forming domain is imported into mitochondria in the absence of ATP. J. Biol. Chem. *263*, 4997–5000.

Chirico, W.J. (1992). Dissociation of complexes between 70 kDa stress proteins and presecretory proteins is facilitated by a cytosolic factor. Biochem. Biophys. Res. Comm. *189*, 1150–1156.

Craig, E.A., Gambill, B.D., and Nelson, R.J. (1993). Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. *57*, 402–414.

Cyr, D.M., Lu, X., and Douglas, M.G. (1992). Regulation of eucaryotic Hsp70 function by a DnaJ homologue. J. Biol. Chem. *267*, 20927–20931.

Cyr, D.M., Stuart, R.A., and Neupert, W. (1993). A matrix ATP requirement for presequence translocation across the inner membrane of mitochondria. J. Biol. Chem. *268*, 23751–23754.

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature *332*, 800–805.

Eilers, M., Hwang, S., and Schatz, G. (1988). Unfolding and refolding of a purified precursor protein during import into isolated mitochondria. EMBO J. *7*, 1139–1145.

Eilers, M., Oppliger, W., and Schatz, G. (1987). Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. EMBO J. *6*, 1073–1077.

Ellis, R.J., and van der Vies, S.M. (1991). Molecular chaperones. Annu. Rev. Biochem. *60*, 321–347.

Gambill, B.D., Voos, W., Kang, P.J., Mao, B., Langer, T., Craig, E., and Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. J. Cell Biol. *123*, 109–117.

Gething, M.-J., and Sambrook, J. (1992). Protein folding in the cell. Nature *355*, 33–45.

Glick, B.S. (1991). Protein import into isolated yeast mitochondria. Methods Cell Biol. *34*, 389–399.

Glick, B.S., Brandt, A., Cunningham, K., Müller, S., Hallberg, R.L., and Schatz, G. (1992a). Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. Cell *69*, 809–822.

Glick, B.S., Wachter, C., Reid, G.A., and Schatz, G. (1993). Import of cytochrome b_2 to the mitochondrial intermembrane space: the tightly folded heme-binding domain makes import dependent upon matrix ATP. Protein Sci. *2*, 1901–1917.

Glick, B., Wachter, C., and Schatz, G. (1991). Protein import into mitochondria: two systems acting in tandem? Trends Cell Biol. *1*, 99–103.

Glick, B.S., Wachter, C., and Schatz, G. (1992b). The energetics of protein import into mitochondria. Biochim. Biophys. Acta *1101*, 249–251.

Glover, L.A., and Lindsay, J.G. (1992). Targeting proteins to mitochondria: a current overview. Biochem. J. *284*, 609–620.

Hachiya, N., Alam, R., Sakasegawa, Y., Sakaguchi, M., Mihara, K., and Omura, T. (1993). A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. EMBO J. *12*, 1579–1586.

Hannavy, K., Rospert, S., and Schatz, G. (1993). Protein import into mitochondria: a paradigm for the translocation of polypeptides across membranes. Curr. Opin. Cell Biol. *5*, 694–700.

Hendrick, J.P., Langer, T., Davis, T.A., Hartl, F.-U., and Wiedmann, M. (1993). Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides. Proc. Natl. Acad. Sci. USA *90*, 10216–10220.

Hurt, E.C., Pesold-Hurt, B., and Schatz, G. (1984). The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. EMBO J. *3*, 3149–3156.

Hwang, S.T., and Schatz, G. (1989). Translocation of proteins across the mitochondrial inner membrane, but not into the outer membrane, requires nucleoside triphosphates in the matrix. Proc. Natl. Acad. Sci. USA *86*, 8432–8436.

Hwang, S.T., Wachter, C., and Schatz, G. (1991). Protein import into the yeast mitochondrial matrix: a new translocation intermediate between the two mitochondrial membranes. J. Biol. Chem. *266*, 21083–21089.

Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A., and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature *348*, 137–143.

Lill, R., Stuart, R.A., Drygas, M.E., Nargang, F.E., and Neupert, W. (1992). Import of cytochrome c heme lyase into mitochondria: a novel pathway into the intermembrane space. EMBO J. *11*, 449–456.

Lithgow, T., Høj, P.B., and Hoogenraad, N.J. (1993). Do cytosolic factors prevent promiscuity at the membrane surface? FEBS Lett. *329*, 1–4.

Manning-Krieg, U.C., Scherer, P.E., and Schatz, G. (1991). Sequential action of mitochondrial chaperones in protein import into the matrix. EMBO J. *10*, 3273–3280.

Martin, J., Mahlke, K., and Pfanner, N. (1991). Role of an energized inner membrane in mitochondrial protein import. J. Biol. Chem. *266*, 18051–18057.

Mattingly, J.R., Youssef, J., Iriate, A., and Martinez-Carrion, M. (1993). Protein folding in a cell-free translation system. J. Biol. Chem. *268*, 3925–3937.

Mayer, A., Lill, R., and Neupert, W. (1993). Translocation and insertion of precursor proteins into isolated outer membranes of mitochondria. J. Cell Biol. *121*, 1233–1243.

Miller, B.R., and Cumsky, M.G. (1991). An unusual mitochondrial import pathway for the yeast cytochrome c oxidase subunit Va. J. Cell Biol. *112*, 833–841.

Miller, B.R., and Cumsky, M.G. (1993). Intramitochondrial sorting of the precursor to yeast cytochrome c oxidase subunit Va. J. Cell Biol. *121*, 1021–1029.

Murakami, H., Pain, D., and Blobel, G. (1988). 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. *107*, 2051–2057.

Murakami, K., Tanase, S., Morino, Y., and Mori, M. (1992). Presequence binding factor-dependent and factor-independent import of proteins into mitochondria. J. Biol. Chem. *267*, 13119–13122.

Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E.A. (1992). The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. Cell *71*, 97–105.

Neupert, W., Hartl, F.U., Craig, E.A., and Pfanner, N. (1990). How do polypeptides cross the mitochondrial membranes? Cell *63*, 447–450.

Neupert, W., and Pfanner, N. (1993). Roles of molecular chaperones in protein targeting to mitochondria. Phil. Trans. R. Soc. Lond. B *339*, 355–362.

Ostermann, J., Horwich, A.L., Neupert, W., and Hartl, F.U. (1989). Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. Nature *341*, 125–130.

Ostermann, J., Voos, W., Kang, P.J., Craig, E.A., Neupert, W., and Pfanner, N. (1990). Precursor proteins in transit through mitochondrial contact sites interact with hsp70 in the matrix. FEBS Lett. *277*, 281–284.

Pfanner, N., and Neupert, W. (1986). Transport of F_1-ATPase subunit beta into mitochondria depends on both a membrane potential and nucleoside triphosphates. FEBS Lett. *209*, 152–156.

Pfanner, N., Pfaller, R., Kleene, R., Ito, M., Tropschug, M., and Neupert, W. (1988). Role of ATP in mitochondrial protein import. J. Biol. Chem. *263*, 4049–4051.

Pfanner, N., Rassow, J., Guiard, B., Söllner, T., Hartl, F.-U., and Neupert, W. (1990). Energy requirements for unfolding and membrane translocation of precursor proteins during import into mitochondria. J. Biol. Chem. *265*, 16324–16329.

Pfanner, N., Tropschug, M., and Neupert, W. (1987). Mitochondrial protein import: nucleoside triphosphates are involved in conferring import-competence to precursors. Cell *49*, 815–823.

Pilon, M., Rietveld, A.G., Weisbeek, P.J., and de Kruijff, B. (1992). Secondary structure and folding of a functional chloroplast precursor protein. J. Biol. Chem. *267*, 19907–19913.

Rassow, J., and Pfanner, N. (1991). Mitochondrial preproteins en route from the outer membrane to the inner membrane are exposed to the intermembrane space. FEBS Lett. *293*, 85–88.

Rothman, J.E. (1989). Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell *59*, 591–601.

Scherer, P.E., Krieg, U.T., Hwang, S.T., Vestweber, D., and Schatz, G. (1990). A precursor protein partly translocated into yeast mitochondria is bound to a 70 kd mitochondrial protein. EMBO J. *9*, 4315–4322.

Schleyer, M., and Neupert, W. (1985). Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes. Cell *43*, 339–350.

Schwarz, E., Seytter, T., Guiard, B., and Neupert, W. (1993). Targeting of cytochrome b_2 into the mitochondrial intermembrane space: specific recognition of the sorting signal. EMBO J. *12*, 2295–2302.

Segui-Real, B., Stuart, R.A., and Neupert, W. (1992). Transport of proteins into the various subcompartments of mitochondria. FEBS Lett. *313*, 2–7.

Simon, S.M., Peskin, C.S., and Oster, G.F. (1992). What drives the translocation of proteins? Proc. Natl. Acad. Sci. USA *89*, 3770–3774.

Skerjanc, I.S., Sheffield, W.P., Randall, S.K., Silvius, J.R., and Shore, G.C. (1990). Import of precursor proteins into mitochondria—site of polypeptide unfolding. J. Biol. Chem. *265*, 9444–9451.

Smith, B.J., and Yaffe, M.P. (1991). A mutation in the yeast heat-shock factor gene causes temperature-sensitive defects in both mitochondrial protein import and the cell cycle. Mol. Cell. Biol. *11*, 2647–2655.

Thorner, J.W., and Paulus, H. (1973). Glycerol and glycerate kinases. In: The Enzymes, vol. VIII, ed. P.D. Boyer, New York: Academic Press, 487–508.

Verner, K. (1993). Co-translational protein import into mitochondria: an alternative view. Trends Biochem. Sci. *18*, 366–371.

Verner, K., and Schatz, G. (1987). Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP. EMBO J. *6*, 2449–2456.

Vestweber, D., and Schatz, G. (1988). Point mutations destabilizing a precursor protein enhance its post-translational import into mitochondria. EMBO J. *7*, 1147–1151.

von Jagow, G., and Klingenberg, M. (1970). Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. Eur. J. Biochem. *12*, 583–592.

Voos, W., Gambill, B.D., Guiard, B., Pfanner, N., and Craig, E. (1993). Presequence and mature part of preproteins strongly influence the dependence of mitochondrial protein import on heat shock protein 70 in the matrix. J. Cell Biol. *123*, 119–126.

Wachter, C., Schatz, G., and Glick, B.S. (1992). Role of ATP in the intramitochondrial sorting of cytochrome c_1 and the adenine nucleotide translocator. EMBO J. *11*, 4787–4794.