Characterization of the Mitochondrial Binding and Import Properties of Purified Yeast F1-ATPase β Subunit Precursor

IMPORT REQUIRES EXTERNAL ATP*

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To better understand the early events of the mitochondrial protein import process, we purified the precursor of the F1-ATPase β subunit (pre-F1β) and examined its import into isolated mitochondria. Import of purified urea-denatured pre-F1β did not require cytosolic factors. However, the period of productive import was prolonged by the addition of reticulocyte lysate, suggesting that cytosolic factors such as molecular chaperones were acting to extend the period of import competence of pre-F1β. Purified pre-F1β bound extensively to both cardiolipin-containing liposomes and to intact mitochondria, indicating that a direct interaction between mitochondrial precursors and the mitochondrial outer membrane surface can occur. The ability to chase this surface-bound pre-F1β into mitochondria suggests that precursors bound to the mitochondrial surface can be maintained in an import competent conformation. Finally, our defined mitochondrial import system was used to characterize the ATP requirements of pre-F1β import in the absence of cytosol. We found a strong requirement for ATP on both sides of the mitochondrial inner membrane, suggesting that one or more previously undetected mitochondrial proteins outside the inner membrane utilize ATP to promote efficient pre-F1β import.

The compartmental organization of eukaryotic cells requires that specific and efficient machineries exist to transport proteins from their site of synthesis across one or more membranes to their final subcellular destination. Although the details of these processes may vary, their common biophysical requirements have led to a conservation of many general features of protein transport across biological membranes. For proteins to be imported into mitochondria, they must be translocated across both the inner and outer membranes to reach the mitochondrial matrix. Most proteins destined for import into mitochondria are synthesized with an NH2-terminal targeting signal having the general features of an amphipathic helix (von Heijne, 1986; Roise and Schatz, 1988; Bedwell et al., 1989). These targeting signals are thought to initiate mitochondrial import by a direct interaction with import receptors on the mitochondrial surface. In Neurospora crassa, outer membrane proteine proteins involved in protein translocation include the protein import receptors MOM19 and MOM72 and the associated MOM22, along with the proteins MOM38, MOM30, MOM8, and MOM7, which are components of the general insertion pore (Kiebler et al., 1990, 1993; Pfaller et al., 1988). Yeast homologs of many of these proteins have also been identified (Moczko et al., 1992). In particular, the yeast proteins Mas70 and ISP42, previously shown to be involved in mitochondrial protein import (Hines et al., 1990; Baker et al., 1990), appear to be homologs of MOM72 and MOM38, respectively. Another low Mr, yeast outer membrane protein, ISP6, has been shown to directly interact with ISP42 (Kassenbrock et al., 1993). Finally, the Mas6 protein of yeast recently became the first component of the inner membrane protein import machinery to be identified (Emtage and Jensen, 1993). Although recent studies have shown that the machineries involved in translocating proteins across each mitochondrial membrane can function independently of one another, they appear to normally interact in a dynamic fashion to achieve simultaneous translocation across both membranes (Segui-Real et al., 1993).

Protein transport across the endoplasmic reticulum membrane appears to proceed by both co- and post-translational mechanisms. In the post-translational pathway, molecular chaperones maintain newly synthesized precursors in a loosely folded import-competent conformation prior to membrane translocation. In contrast, a ribonucleoprotein complex termed signal recognition particle has been shown to facilitate the co-translational movement of secretory proteins across the endoplasmic reticulum membrane (Portitz et al., 1990). Other studies suggest that co- and post-translational pathways for protein transport into mitochondria may also exist. Recently, it was suggested that bulk mitochondrial protein import proceeds primarily by a co-translational mechanism in vitro (Fujiki and Vernier, 1993). Presently, the specific cytosolic factors (if any) required to mediate this process are undefined. In contrast, the molecular chaperones Hsp70 (Murakami et al., 1988) and YDJ1 (Caplan et al., 1992) appear to stimulate post-translational mitochondrial protein import by maintaining precursors in a loosely folded, import competent conformation prior to membrane translocation. The importance of such an unfolded conformation is supported by the observation that urea denaturation stimulates the import of many precursors. In other cases, however, urea denaturation alone does not appear to be sufficient to promote efficient mitochondrial import in vitro. For example, the import of purified, urea-denatured pre-ornithine carbamoyltransferase has been shown to require a reticulocyte lysate (RL) factor called presequence binding factor, which appears to bind specifically to the presequence of pre-ornithine carbamoyltransferase (Murakami and Mori, 1990). Whether

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‡ The abbreviations used are: RL, reticulocyte lysate; PMSF, phenylmethylsulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; Cl, cardiolipin; PG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac(1-glycerol).
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presequence binding factor represents another molecular chaperone or some other factor, such as a soluble receptor, remains to be determined. In contrast, cytosolic factors were not found to be required for the import of purified urea-denatured pre-adrenodoxin (Iwahashi et al., 1992) or a purified urea-denatured chimeric protein (Becker et al., 1992). These results demonstrate that individual precursor proteins appear to have differing cytosolic requirements for their import into mitochondria. These differences may correspond to unique properties associated with each protein.

To better understand the early events in the mitochondrial protein import pathway, we have purified the precursor of the F1-ATPase β subunit (pre-F1β) and characterized the specific conditions required for its import into isolated mitochondria. Import of urea-denatured pre-F1β did not require cytosolic factors, but was increased 2-fold by the addition of RL. Purified pre-F1β bound extensively to liposomes and to intact mitochondria, demonstrating that a direct interaction between mitochondrial precursors and the mitochondrial outer membrane can occur. Finally, our defined mitochondrial import system was used to characterize the ATP requirement of pre-F1β import in the absence of cytosol. Our results indicated a strong requirement for ATP on both sides of the mitochondrial inner membrane, suggesting that one or more previously undetected mitochondrial factors outside the inner membrane utilize ATP to promote efficient pre-F1β import.

**MATERIALS AND METHODS**

**Construction of Pre-F1β Expression Plasmid—**A 1.6-kilobase HindIII fragment containing the intact ATP2 gene (Bedwell et al., 1987) was subcloned into the HindIII site of M13mp18, yielding the construct DBM13. Site-directed mutagenesis was carried out using the oligonucleotide 5'-AAAAATTAAAAAAACATCAAGTTGTTGCG-3' to create a unique NdeI restriction site (underlined) which overlapped the translation initiation codon of the ATP2 gene. The HindIII fragment containing the NdeI site was subcloned into the T7 promoter expression vector pET3a (Studier et al., 1990). The pET3a/ATP2 construct was designated pDB288.

**Expression and Purification of Pre-F1β—**E. coli strain BL21(DE3) transformed with pDB288 was grown in minimal medium containing 25 µg/ml ampicillin at 37 °C. When the culture density reached 0.6 A465 units/ml, pre-F1β expression was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. 5 min later 0.5 µM of [35S]methionine/cysteine (DuPont NEN) was added to 1 ml of the culture, and cultivation was continued for 45 min at 37 °C. Cells were collected by centrifugation at 16,000 × g for 2 min at 4 °C and washed with 1 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE). The cell pellet was resuspended in 0.1 ml of lysate buffer containing 1 mM mg/lysozyme, 50 mM EDTA, 1 mM PMSF, and 10 µg/ml Tris-HCl, pH 7.5. Cells were lysed by six freeze/thaw cycles. Then 0.9 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl (TEN) buffer containing 10 mM MgCl2, 10 µg/ml DNase I, 1 mg/ml deoxycholate, and 1 µg/ml PMSF was added to the lysate. After 10 min of incubation on ice, the lysate was centrifuged at 16,000 × g for 10 min at 4 °C. The pellet was treated with 1 ml of TEN buffer containing 1% Triton X-100, 1 mM PMSF for 1 h at 4 °C, centrifuged at 16,000 × g for 10 min at 4 °C, and washed with 1 ml of TE buffer containing 1 mM PMSF. Finally, the pellet was solubilized in 50 µl of urea buffer (8 M urea, 20 mM Tris, 1 mM EDTA, 1 mM PMSF), pH 7.5, for 1 h at 4 °C, and insoluble debris was removed by centrifugation at 16,000 × g for 20 min at 4 °C. The supernatant containing purified urea-denatured pre-F1β was stored in aliquots at –80 °C.

**In Vitro Synthesis of Pre-F1β—**The DNA template for in vitro coupled transcription/translation of pre-F1β was pDB95, which contained a 1.6-kilobase HindIII fragment encoding the ATP2 gene (Bedwell et al., 1987). The HindIII site of plasmid pSP64 was used to enhance the ATP2 gene transcription/translation reaction containing 57% rabbit reticulocyte lysate, 25 mM Tris-HCl, pH 7.5, 1.6 mM magnesium acetate, 0.4 mM spermidine, 0.4 mM dithiothreitol, 0.5 mM each of ATP, CTP, GTP, and UTP, 20 µM amino acids (minus methionine), 0.6 µM/ml [35S]methionine/cysteine, 800 units/ml RNAsin, 40 µg/ml template DNA, and 400 units/ml SP6 RNA polymerase. The reaction was incubated for 2 h at 30 °C and then stored frozen at –80 °C until use.

**In Vitro Mitochondrial Protein Import—**Mitochondria were prepared using yeast strain D273-10B essentially as described (Glaser and Cumming, 1989). The typical 0.1-ml import reaction contained 10 mM MOPS, pH 7.2, 2 mg/ml BSA (fatty acid-free), 250 µM sucrose, 1 mM ATP, 10 mM succinate, 86 mM KOAc, 0.9 mM MgOAc, 1 mM diethiothreitol, 25 mM creatine phosphate, 4 mg/ml creatine phosphokinase, and 0.5 mM mitochondrial. For ATP depletion experiments, both ATP and the ATP regenerating system were omitted, and the reaction mixture was pre-treated with 1 unit/ml apyrase for 10 min on ice. The import reaction was started by the addition of 2.5 µl of purified urea-denatured [35S]-labeled pre-F1β at 25 °C, resulting in a final urea concentration of 0.2 M. The reaction was stopped with 0.9 ml of ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) containing 1 µg/ml valinomycin. Parallel samples were treated with 50 µg/ml trypsin for 15 min on ice, then stopped by the addition of 500 µg/ml trypsin inhibitor. The samples were analyzed by SDS-PAGE and autoradiography, followed by quantitation using a PhosphorImager (Molecular Dynamics).

**Preparation of Liposomes—**Multilamellar phospholipid liposomes were prepared by the method of Bangham et al. (1965). Bovine heart phosphatidylcholine, bovine heart phosphatidylethanolamine, bovine heart cardiolipin (CL), and synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-1-glycerol (PG) were all purchased from Avanti Polar Lipids. Phospholipid films (4 µmol) were resuspended in 1 ml of binding buffer (86 mM KOAc, 0.9 mM MgOAc, 10 mM MOPS, pH 7.2) by vortexing for 2 min. Liposomes were washed three times with binding buffer containing 1 mg/ml BSA by centrifugation at 16,000 × g for 20 min. When liposomes were prepared for the import assay, the binding buffer was supplemented with 250 mM sucrose. If not indicated, the typical (15% CL) liposomes were composed of 45 mol % phosphatidylcholine, 30 mol % phosphatidylethanolamine, 15 mol % CL, and 10 mol % PG.

**Binding Assays—**Binding assays were initiated by the addition of 2.5 µl of urea-denatured pre-F1β to the suspension of liposomes pre-equilibrated at 25 °C in binding buffer containing 1 mg/ml BSA. After 20 min, binding was terminated by a 10-fold dilution in ice-cold binding buffer. The liposomes were immediately re-isolated by centrifugation at 16,000 × g for 20 min at 4 °C and washed with 0.5 µl of binding buffer containing 1 mg/ml BSA. The washed liposome pellet was resuspended in binding buffer, and the import reaction was initiated for ATP-dependent mitochondrial protein import assays. The pre-F1β bound to liposomes was expressed as total pelletable pre-F1β, corrected for nonspecific aggregation (in the absence of liposomes, <5% of input). To assay binding to intact mitochondria, the binding reaction was carried out in protein import buffer supplemented with 1 µg/ml valinomycin.

**RESULTS**

**Expression and Purification of Pre-F1β—**Pre-F1β was over-produced in E. coli strain BL21(DE3) using the T7 RNA polymerase expression system (Studier et al., 1990). Using this system, pre-F1β was synthesized in large quantities (20–50 mg/liter of culture) in both minimal and rich media. The recombinant protein was found to accumulate in inclusion bodies, which aided in its purification. Following cell lysis, the inclusion bodies were recovered by centrifugation and purified from contaminating E. coli proteins by successive washes in buffers containing deoxycholate and Triton X-100 (see “Materials and Methods”). The final pellet was solubilized in 8 M urea and was found to contain highly purified pre-F1β (Fig. 1). [35S]-Labeled pre-F1β was also prepared using this system, and the resulting radiolabeled denatured precursor was suitable for use in in vitro import studies.

**Import of Purified Pre-F1β into Isolated Mitochondria—**The urea-solubilized [35S]-labeled pre-F1β was found to import efficiently into isolated yeast mitochondria in the absence of any cytosolic proteins (Fig. 2A). Import reached a plateau within 150 min, when 30% of the precursor was imported and processed to the mature form. These results indicate that denatured pre-F1β does not require cytosolic factors to undergo mitochondrial import. However, to exclude the possibility that cytosolic factors that could facilitate pre-F1β import had copurified with either the radiolabeled pre-F1β or the isolated mitochondria, both precursor and mitochondria were purified more extensively. To further purify pre-F1β, the urea-solubilized precursor preparation was resolved by SDS-PAGE and...
Fig. 1. Purification of pre-F1β overproduced in E. coli. Purification steps were analyzed by 8% SDS-PAGE: lane 1, total cell homogenate; lane 2, 16,000 x g pellet; lane 3, Triton X-100-washed pellet; lane 4, Tris-EDTA-washed pellet; lane 5, urea-solubilized pellet. The relative mobility of molecular mass standards are indicated on the left in kDa.

electroeluted from gel slices. To remove peripherally associated proteins, mitochondria were subjected to a salt wash with 0.25 M KCl. Both the rate of import and the total yield of imported protein using gel-purified pre-F1β and salt-washed mitochondria were similar to the results shown in Fig. 2A (data not shown), excluding the possibility that contaminating proteins facilitated the import of pre-F1β. Thus, urea-denatured pre-F1β does not require cytosolic factors for import. These findings are consistent with results reported previously for purified adrenodoxin (Iwahashi et al., 1992) and a purified fusion protein that joined portions of cytochrome b2 and F1β (Becker et al., 1992).

When rabbit RL was added to the import reaction, the yield of imported F1β was increased (Fig. 2A). Under these conditions, 70–80% of added precursor was routinely imported and processed within 30 min. The RL stimulation does not appear to result from a general increase in protein concentration because BSA alone did not elicit this effect. RL has been reported to contain a number of factors that stimulate mitochondrial protein import. Some of these factors, such as Hsp70, have been reported to stimulate mitochondrial protein import by helping to maintain precursors in an import-competent conformation. To better understand the mechanism of RL stimulation of pre-F1β import, we examined in greater detail the effect of RL on the initial kinetics of pre-F1β import (Fig. 2B). We found that the initial rate of pre-F1β import was identical in the presence or absence of RL, indicating that RL does not stimulate import by increasing the absolute number of pre-F1β molecules undergoing import during the initial stages of the reaction. Rather, the data indicate that the RL-mediated stimulation of pre-F1β import is caused primarily by an extension of the total time period during which import occurs. This result is consistent with a stimulation by molecular chaperones, which would act to prolong the window during which extramitochondrial pre-F1β is maintained in a loosely folded conformation compatible with mitochondrial import.

To exclude the possibility that RL exerts its stimulatory effect by extending the import competence of mitochondria rather than the import competence of pre-F1β, an import reaction was initiated with urea-denatured precursor in the absence of RL. After import began to level off, an additional aliquot of pre-F1β was added (Fig. 2C). The rate of import following precursor re-addition was almost identical to the initial rate of pre-F1β import, indicating that the mitochondria remained capable of importing unfolded pre-F1β with similar kinetics throughout the first 20 min of import in the absence of RL. These results confirm that RL factors stimulate pre-F1β

![Fig. 2. Time course of pre-F1β import into isolated mitochondria.](image-url)

**A**. pre-F1β import was carried out in the presence of 14% RL S100 (closed circles) or a corresponding concentration of BSA (open circles). **B**, time course of purified pre-F1β import during the first 5 min in the presence of 14% RL S100 (closed circles) or a corresponding concentration of BSA (open circles). **C**, sequential addition of purified pre-F1β during mitochondrial import. The time course of pre-F1β import initiated at time 0 is indicated by the open circles. After the reaction had proceeded for 10 min, it was divided into two aliquots, and 30 s later a fresh aliquot of urea-denatured pre-F1β was added to one tube (closed circles).
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import primarily by extending the period of import competence of purified pre-F$_{1}$P during the import reaction. Most importantly, no evidence was found of RL factors stimulating the initial rate of import of urea-denatured pre-F$_{1}$P.

**RL Prevents Binding of Pre-F$_{1}$P to Cardiolipin-containing Liposomes**—To further investigate the early events in mitochondrial protein import, multimellar phospholipid liposomes were used to simulate the initial interactions between pre-F$_{1}$P and the mitochondrial surface. A previous study reported that RL-translated mitochondrial precursors bind to multimellar liposomes having a phospholipid composition similar to that of the mitochondrial outer membrane (Ou et al., 1988). Cardiolipin (CL), an acidic phospholipid enriched in mitochondrial membranes, was found to be required for efficient precursor binding to those vesicles. Since the yeast mitochondrial outer membrane, inner membrane, and close contact sites have been reported to contain 6, 16, and 17 mol % of CL, respectively (Simbeni et al., 1991), we examined the ability of liposomes having roughly the CL content of close contact sites (15 mol %) to bind pre-F$_{1}$P. Purified pre-F$_{1}$P was found to efficiently bind to CL-containing liposomes (Fig. 3A). However, when RL (14% final concentration) was included in the binding assay, pre-F$_{1}$P did not bind to the liposomes. This indicates that RL factors interact with either the precursor or liposomes and prevents binding of the precursor protein. This effect is not simply due to high protein concentrations, since pre-F$_{1}$P binding to CL-containing liposomes was not prevented by similar amounts of BSA. This finding is not consistent with the results of binding studies carried out with other precursors, since it has been shown that several RL-translated precursors are able to bind CL-containing liposomes (Ou et al., 1988). Therefore, we directly tested RL-translated pre-F$_{1}$P (0.7% final RL concentration in the binding reaction) for its ability to bind CL-containing liposomes. Little or no binding was observed (<7% of input), confirming that RL-translated pre-F$_{1}$P does not efficiently bind to CL-containing liposomes.

The requirement for CL to promote pre-F$_{1}$P binding to membranes was tested using liposomes in which an equimolar amount of phosphatidylglycerol (PG) was substituted for CL, with the relative composition of the other phospholipids held constant. The binding of pre-F$_{1}$P to liposomes containing PG was almost 6-fold lower than to liposomes containing CL (Fig. 3B). Since the net charge of CL is $-2$ and PG is $-1$, a trivial explanation for the difference in liposome binding is the difference in net charge between these liposomes. However, similar pre-F$_{1}$P binding was observed using liposomes containing 7 mol % CL (data not shown), indicating that the CL-dependent stimulation of pre-F$_{1}$P binding to liposomes is not simply due to net charge effects.

**Effect of Liposomes on Import of Purified Pre-F$_{1}$P into Mitochondria**—Since CL-containing liposomes efficiently bound purified pre-F$_{1}$P, we asked whether they could inhibit pre-F$_{1}$P import into mitochondria. When purified urea-denatured pre-F$_{1}$P was added to an import reaction in the absence of RL, the addition of increasing amounts of CL-containing liposomes decreased pre-F$_{1}$P import 3-fold over the range of phospholipid concentrations used (Fig. 4A). When RL was added, the total amount of purified pre-F$_{1}$P that was imported increased 2-fold, and import was completely insensitive to liposome inhibition. Thus, the RL-mediated stimulation of pre-F$_{1}$P import by liposomes, apparently by preventing the precursor molecules from being nonproductively sequestered on the surface of the liposomes. When RL-translated pre-F$_{1}$P was added to an import reaction in the presence of CL-containing liposomes (0.7% RL final concentration), we found that a similar liposome concentration reduced pre-F$_{1}$P import only 1.6-fold (Fig. 4B). The addition of RL (to 14% final concentration) did not further stimulate import of the RL-translated pre-F$_{1}$P, but did abolish the low level of liposome-mediated import inhibition. Although we cannot exclude the possibility that cytosolic factors act to specifically target the precursor to mitochondria, the simplest interpretation of these results is that molecular chaperones act to prevent the nonproductive binding of pre-F$_{1}$P to liposomes.

To determine when RL must interact with pre-F$_{1}$P to exert its stimulatory effect, we asked whether RL could stimulate import after pre-F$_{1}$P had been bound to the mitochondrial surface. To carry out this experiment, pre-F$_{1}$P was bound to mitochondria in the absence of ATP, a condition which prevents mitochondrial protein import (Chen and Douglas, 1987; Pfanner et al., 1987; Eilers et al., 1987; see below). Mitochondria and associated pre-F$_{1}$P were re-isolated by centrifugation for 10 min at 4°C, resuspended in import buffer, and import was initiated by the addition of ATP in the presence or absence of RL (Fig. 5). Within 20 min, 33% of the bound pre-F$_{1}$P was imported in the
increasing concentrations of liposomes (expressed as total phospholipid BSA) presence concentration) were incubated for import of purified urea-denatured pre-F,P (reactions contained a final concentration of 0.7% RL (labeled tochondria remained competent for import into freshly added import kinetics and RL stimulation (data not shown). Further-
increase). This indicated that although precursors bound to the absence of RL. When the import reaction was carried out in the presence of RL, 40% of the pre-F,P was imported (only a 20% increase). This indicated that although precursors bound to the mitochondrial surface remain capable of import, they are largely insensitive to the stimulatory effects of RL. Control experiments confirmed that re-isolated mitochondria were import competent, since freshly added pre-F,P exhibited normal import kinetics and RL stimulation (data not shown). Furthermore, pre-F,P treated under the same conditions without mitochondria remained competent for import into freshly added mitochondria and was subject to RL stimulation, indicating that the precursor retained import competence in the absence of mitochondria. These results suggest that RL factors must bind precursors prior to their binding to the surface of mitochondria for the RL-mediated stimulation of pre-F,P import to occur. The bound precursor that is imported must represent the fraction of precursors that were bound either in a reversible form (which should be susceptible to RL-mediated stimulation) or in an immediately productive manner, possibly directly associated with import receptors or bound to the membrane surface at (or near) close contact sites.

Role of Trypsin-sensitive Mitochondrial Surface Proteins in Pre-F,P Binding and Import—Previous studies have shown that receptor proteins on the surface of mitochondria mediate the binding of a number of mitochondrial precursors. Mitochon-
drial outer membrane proteins reported to function as receptors include Mas70 and p32 in yeast (Hines et al., 1990; Pain et al., 1990) and MOM19 and MOM72 in Neurospora (Sollner et al., 1989, 1990). Previous studies have shown that the mitochondrial import receptors MOM19 and MOM72 are extremely sensitive to mild protease treatment, whereas components of the general insertion pore complex are resistant (Sollner et al., 1989, 1990; Kiebler et al., 1990, 1993). Therefore, mitochondria were treated with trypsin, and pre-F,P import was carried out under conditions in which import was linear with respect to both time and the concentration of mitochondria. Fig. 6A shows the import of purified pre-F,P into mitochondria in the presence or absence of RL (14% final concentration) as a function of the extent of trypsin treatment of the mitochondria. As expected, the absolute level of import in both cases decreased with increasing trypsin concentration and eventually approached a plateau of residual import. However, while the absolute import efficiencies (+RL) differed, the relative import rates show very similar trypsin sensitivities (Fig. 6B). This indicates that proteins on the mitochondrial surface involved in pre-F,P import function equally well in the presence or absence of cytosolic factors, suggesting that soluble factors do not directly interact with trypsin-sensitive mitochondrial surface proteins during pre-F,P import.

We next examined the role of trypsin-sensitive factors in the initial binding of purified pre-F,P to intact import-competent mitochondria. As controls, the binding of the RL-translated precursor of alcohol dehydrogenase III (pre-ADHIII) and pre-F,P were also examined. To block precursor import, valinomycin was used to dissipate the potential across the inner mitochondrial membrane. In the absence of trypsin treatment, we found that 56% of the pre-ADHIII bound to mitochondria in the presence of 0.5% RL (Fig. 7A). The addition of 25 °C to allow pre-F,P binding. The mitochondria and associated pre-F,P were then re-isolated by centrifugation for 10 min at 4 °C, and import of bound pre-F,P was assayed in the presence of 1 mM ATP and an ATP-regenerating system. Either 14% RL S100 (closed circles) or a comparable amount of BSA (open circles) were included during the import reaction as indicated.

Fig. 4. In vitro mitochondrial import of purified pre-F,P in the presence of CL-containing liposomes. Import reactions containing increasing concentrations of liposomes (expressed as total phospholipid concentration) were incubated for 30 min in the presence of either 14% RL S100 (closed bars) or a corresponding amount of BSA (open bars). A, import of purified urea-denatured pre-F,P. B, import of RL-translated pre-F,P (reactions contained a final concentration of 0.7% RL (labeled BSA) or 14% (labeled RL)).
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Fig. 6. Effect of trypsin treatment of mitochondria on import of purified pre-F₁β. A, mitochondria (2 mg/ml) were pretreated with various concentrations of trypsin (expressed as μg of trypsin/mg of mitochondrial protein) for 10 min on ice, diluted 10-fold in SEM containing 200 μg/ml trypsin inhibitor and re-isolated by centrifugation. They were washed once with SEM containing 100 μg/ml trypsin inhibitor, and import was then carried out in the presence of either 14% RL S100 or a corresponding concentration of BSA for 30 min. B, absolute import efficiencies were normalized to trypsin-untreated import levels obtained in the presence of either 14% RL S100 (closed circles) or a corresponding concentration of BSA (open circles).

Fig. 7. Effect of trypsin treatment of mitochondria on pre-F₁β binding. Mitochondria (2 mg/ml) were either untreated (A) or treated (B) with 10 μg of trypsin/mg of mitochondrial protein for 10 min on ice. They were diluted 10-fold in SEM containing 200 μg/ml trypsin inhibitor and re-isolated by centrifugation. They were washed once with SEM containing 100 μg/ml trypsin inhibitor, and binding was then carried out in the presence of 1 μg/ml valinomycin and either 14% RL S100 (closed bars) or the corresponding amount of BSA (open bars).

al., 1984). In contrast, a large fraction (91%) of the purified urea-denatured pre-F₁β bound to mitochondria. Again, the addition of RL to a final concentration of 14% caused a severe reduction in pre-F₁β binding (to only 7.4% of input). These results demonstrate that cytosolic factors present in RL block pre-F₁β binding, a result not observed with either pre-ADHIII or a number of other mitochondrial precursors. These data are also consistent with the observed pre-F₁β binding to CL-containing liposomes, indicating that the liposome model accurately reflects pre-F₁β binding to the mitochondrial surface.

When mitochondria were treated with the maximal trypsin concentration (10 μg/ml) used in the previous experiment, 25% of RL-translated pre-ADHIII bound to mitochondria, and the further addition of RL slightly reduced pre-ADHIII binding to 17% of input (Fig. 7B). However, only 2.8% of RL-translated pre-F₁β bound to trypsin-treated mitochondria, and the further addition of RL decreased this low level of binding even further. In contrast, 45% of purified denatured pre-F₁β bound to trypsin-treated mitochondria, and this binding was abolished by the addition of RL. In each case, precursor binding was reduced approximately 50% when mitochondria were treated with trypsin. These results indicate that a significant portion of pre-F₁β binds directly to the outer membrane surface as is observed with CL-containing liposomes.

ATP Requirement for Import of Purified Pre-F₁β into Mitochondria in the Absence of Cytosolic Factors—We next examined the ATP requirements for pre-F₁β import in the absence of cytosolic factors. The conditions used to control ATP levels inside and outside mitochondria were similar to those described previously (Wachter et al., 1992; Glick et al., 1992). To examine the importance of external ATP, isolated mitochondria were preincubated with carboxyatractyloside to inhibit the ATP/ADP translocator, and then, prior to import, external ATP was either depleted by apyrase, or both ATP and an ATP generating system were added. In the experiment shown in Fig. 8A, 16.4% of the input pre-F₁β was imported under standard import conditions. In the presence of carboxyatractyloside, 7.2% of the pre-F₁β was imported, indicating that the addition of this inhibitor of the ATP/ADP translocator alone caused a 2-fold drop in pre-F₁β import. Surprisingly, when external ATP was specifically depleted in the presence of carboxyatractyloside, only a small residual level of the total pre-F₁β (0.8%) was imported. This 9-fold drop in import efficiency indicates that external ATP is required for the import of pre-F₁β, even in the absence of cytosolic factors. To exclude the unlikely possibility that cytosolic factors (such as molecular chaperones) had co-fractionated with the mitochondria and were binding pre-F₁β, mitochondria
was carried out. For boxyatractyloside and then with apyrase. Mitochondria were re-isolated and resuspended in buffer containing both oligomycin and carboxyatractyloside to block the exchange of ATP across the inner mitochondrial membrane. The mitochondria were then re-isolated and resuspended in import buffer containing both oligomycin and carboxyatractyloside, either in the absence or presence of ATP. As a control, mitochondria were treated with carboxyatractyloside and then apyrase (no oligomycin), followed by re-isolation of the mitochondria and resuspension in import buffer containing carboxyatractyloside, ATP, and an ATP-regenerating system to control for the effect of carboxyatractyloside treatment on pre-F$_1$β import. Under conditions where ATP was present on both sides of the inner membrane, the level of pre-F$_1$β import was 12.7%, again 2-fold below the import obtained under normal import conditions (Fig. 8B). Under conditions where ATP was only present outside of mitochondria, import was 10-fold lower, with just 1.3% of the pre-F$_1$β being imported. Finally, when no ATP was present, import was completely abolished. These results demonstrate that ATP is required in the mitochondrial matrix for efficient pre-F$_1$β import and are similar to those obtained previously using other precursors (Hwang and Schatz, 1989; Pfanner et al., 1990).

To determine more precisely the intramitochondrial location of the imported pre-F$_1$β in these experiments, mitoplasts were generated in the presence of proteinase K (Fig. 8C). In the presence of ATP on both sides of the inner mitochondrial membrane (plus carboxyatractyloside), the level of imported pre-F$_1$β was 5.1%, again 2-fold lower than import found in mitoplasts under normal import conditions. This level of import indicated that in the presence of ATP on both sides of the inner mitochondrial membrane, imported pre-F$_1$β had efficiently translocated across the inner membrane and into the matrix space. In contrast, the amount of pre-F$_1$β found in mitoplasts in the absence of internal ATP (0.18%) was 28-fold lower than was found in mitoplasts in the presence of both internal and external ATP, again indicating that internal ATP is required for efficient pre-F$_1$β import. These results also suggest that the small amount of pre-F$_1$β imported in the absence of internal ATP accumulated primarily in the intermembrane space. We conclude from these experiments that ATP is required on both sides of the mitochondrial inner membrane for the efficient import of purified pre-F$_1$β, even in the absence of cytosol. Apparently, ATP outside the inner membrane is utilized by mitochondrial proteins either in the outer membrane or the intermembrane space during pre-F$_1$β import.

**DISCUSSION**

Our results indicate that purified urea-denatured pre-F$_1$β is competent for relatively efficient mitochondrial import in the absence of cytosolic factors. Apparently, denaturation of the precursor prior to its addition to the import reaction mimics either a co-translational mode of import or a molecular chaperone-mediated pathway that does not require additional factors. Cytosol-free import has also been reported for purified urea-denatured adrenodoxin precursor (Iwahashi et al., 1992) and resuspended in import buffer containing both carboxyatractyloside and ATP, and import was carried out. C, intramitochondrial localization of imported pre-F$_1$β in the absence of internal ATP. Mitochondria were treated as described in B, and import was carried out. Following the import reaction, mitochondria were diluted 10-fold into buffer lacking sorbitol to generate mitoplasts.
and a purified urea-denatured fusion protein containing the matrix-targeting signal of cytochrome b$_2$ fused to amino acids 51–325 of the N. crassa F$_1$-F$_0$ (representing roughly one-half of the latter protein) (Becker et al., 1992). In contrast, Mori and co-workers (Murakami and Mori, 1990) found that purified, urea-denatured pre-ornithine carbamoyltransferase alone was not competent for import into isolated mitochondria, and they went on to purify a factor from RL designated praseque binding factor that restored import of pre-ornithine carbamoyltransferase. Together, these results suggest that the specific cytosolic requirements for import may vary between precursors, probably as a function of the disposition of each precursor to fold into an import-incompetent conformation. Distinct cytosolic requirements may also be reflected in the affinity of different precursors for molecular chaperones. In this light, the inability to demonstrate pre-F$_1$-F$_0$ binding to mitochondria in the presence of RL (this study; Zwizinski et al., 1984) may simply indicate that Hsp70 efficiently recognizes nascent (or unfolded) pre-F$_1$-F$_0$ as a substrate, thereby reducing its association with the mitochondrial surface. Additional studies to examine the effects of purified Hsp70 on purified pre-F$_1$-F$_0$ binding and import are currently underway.

We found that the addition of cytosol to the import reaction stimulated the total yield of imported pre-F$_1$-F$_0$ 2-fold. This stimulation appeared to result from an extension of the total time during which import occurred, rather than an increase in the rate of import. This result is consistent with a mechanism in which cytosolic factors (such as molecular chaperones) prolong the import competence of the precursor. Molecular chaperones are thought to stimulate protein transport across intracellular membranes by preventing misfolding and aggregation. Our results indicate that cytosolic factors in RL reduce the amount of pre-F$_1$-F$_0$ bound to either CL-containing membranes or mitochondria. Since we also found that RL is largely unable to stimulate pre-F$_1$-F$_0$ import after the precursor is bound to mitochondria, it is likely that these factors (presumably molecular chaperones) bind precursors in solution and act primarily to maintain their loosely folded conformation prior to their association with mitochondria. However, since a significant portion of pre-F$_1$-F$_0$ bound to mitochondria maintained its import competence during the time required to re-isolate the mitochondria, it appears that the interaction between the precursor and the mitochondrial surface itself can also act to maintain the precursor in an import-competent condition. Similarly, Schatz and co-workers (Eilers et al., 1988) found that a CoxIV-DHFR fusion protein unfolded as it bound to the mitochondrial surface and suggested that this interaction represented a true import intermediate in the import pathway.

Mitochondrial import receptors have been shown to be important for the import of a number of precursors (Sollner et al., 1989, 1990; Hines et al., 1990; Hines and Schatz, 1993). However, the exact function(s) mediated by these receptors has not yet been rigorously determined. Although it is generally assumed that receptors directly mediate precursor binding, other interactions may also be important for this process. For example, a growing body of evidence suggests that precursors bind directly to the membrane surface during their early interactions with mitochondria. In this study we found that pre-F$_1$-F$_0$ binds directly to the surface of liposomes, or mitochondria, and similar results have been obtained for other mitochondrial precursor proteins (Ou et al., 1988). These findings are also consistent with previous studies indicating that mitochondrial presequence peptides can insert efficiently into both natural and artificial membrane surfaces independently of import receptors (Roise, 1992; Maduke and Roise, 1993). We also found that pre-F$_1$-F$_0$ binding to liposomes is stimulated by CL, as was shown for the precursors of both adrenodoxin and malate dehydrogenase (Ou et al., 1988). The fact that this phospholipid is greatly enriched in mitochondrial membranes over other cellular membranes may provide additional specificity for the initial precursor interaction with the mitochondrial surface. Taken together, these results suggest that precursor binding to the membrane occurs during mitochondrial protein import and represents a natural intermediate in this process.

The energy requirements for mitochondrial protein import have been reported to include a membrane potential across the inner membrane and ATP both inside and outside the organelle. One role proposed for ATP in the import process is to facilitate the function of molecular chaperones both in the cytosol and in the mitochondrial matrix (Chen and Douglas, 1987; Pfanner et al., 1987; Eilers et al., 1987; Hwang and Schatz, 1989; Pfanner et al., 1990). However, this view would suggest that import of a denatured precursor in the absence of cytosol (and molecular chaperones) may be independent of external ATP. Our results suggest that this assumption is incorrect for pre-F$_1$-F$_0$, since the depletion of ATP either inside or outside of mitochondria caused a 10-fold drop in the import efficiency of pre-F$_1$-F$_0$. Since it has been reported that purified urea-denatured adrenodoxin does not require external ATP for import (Hines and Schatz, 1993), it is possible that ATP is being used by a component of the translocation apparatus that is not required for the import of all precursors. Currently, no such ATP-requiring protein in the mitochondrial outer membrane or intermembrane space has been identified. However, although it has been reported that ATP is not required for precursor binding to receptors on the mitochondrial surface (Pfanner et al., 1990), it is possible that the release of a bound precursor from the receptor, or the function of MOM22 or the general insertion pore may require ATP hydrolysis. Further studies are required to identify this ATP-dependent import factor.

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