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Early Events in the Transport of Proteins into Mitochondria

IMPORT COMPETITION BY A MITOCHONDRIAL PRESEQUENCE*

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Studies with a synthetic presequence peptide, $F_1\beta$1–20, corresponding to the NH_2-terminal 20 amino acids of the F_1-ATPase β-subunit precursor ($pF_1\beta$) show that although this peptide binds avidly to phospholipid bilayers it does not efficiently compete for import of full-length precursor into mitochondria, $K_i \cong 100$ μM (Hoyt, D. W., Cyr, D. M., Gierasch, L. M., and Douglas, M. G. (1991) *J. Biol. Chem.* 266, 21693–21699). Herein we report that longer $F_1\beta$ presequence peptides $F_1\beta$1–32+2, $F_1\beta$1–32SQ+2, and $F_1\beta$21–51+3 compete for mitochondrial import at 1000-, 250-, and 25-fold lower concentrations, respectively, than $F_1\beta$1–20. A longer peptide, $F_1\beta$1–51+3, was no more effective as an import competitor than $F_1\beta$1–32+2. Both minimal length and amphiphilic character appear required in order for $F_1\beta$ peptides to block mitochondrial import. Import competition by longer $F_1\beta$ peptides seems to occur at a step common to all precursors since they blocked import of precursors to F_1-ATPase α- and β-subunits and the ADP/ATP carrier protein. Dissipation of membrane potential ($\Delta\psi$) across the inner mitochondrial membrane is observed in the presence of $F_1\beta$-peptides, but this mechanism alone does not account for the observed import inhibition. $F_1\beta$1–32+2 and 21–51+3 block import of $pF_1\beta$ 100% at peptide concentrations which dissipate $\Delta\psi$ less than 25%. In contrast, experiments with valinomycin demonstrate that when mitochondrial $\Delta\psi$ is reduced 25% import of $pF_1\beta$ is inhibited only 25%. Therefore, at least 75% of maximal import inhibition observed in the presence of $F_1\beta$1–32+2 and $F_1\beta$21–51+3 does not result from dissipation of $\Delta\psi$. Import inhibition by $F_1\beta$-peptides is reversible and can be overcome by increasing the amount of full-length precursor in import reactions. $F_1\beta$ presequence peptides and full-length precursor are therefore likely to compete for a common import step. Presequence dependent binding of $pF_1\beta$ to trypsin-sensitive elements on the outer mitochondrial membrane is insensitive to inhibitory concentrations of $F_1\beta$ presquence peptide. We conclude that import inhibition by $F_1\beta$ presequence peptides is competitive and occurs at a site beyond initial interaction of precursor proteins with mitochondria.

Most mitochondrial proteins are synthesized on cytoplasmic polyribosomes and must be imported either co- or post-translationally into the appropriate suborganellar compartment. Targeting information is typically specified by a stretch of amino acids present at the amino terminus of precursor proteins, the presequence, which is usually cleaved by a metalloprotease upon entry into the mitochondrial matrix (for reviews see Hartl *et al.*, 1989; Pfanner and Neupert, 1990; Verner and Schatz, 1988). Gene fusion studies demonstrate that the presequence alone is necessary and sufficient to direct mitochondrial import of both authentic precursors and non-mitochondrial passenger proteins (Hurt *et al.*, 1984; Horwich *et al.*, 1985; Emr *et al.*, 1986). However, presequences contain no consensus sequence for recognition by the import apparatus (von Heijne, 1986). Instead, sequence analysis of mitochondrial targeting signals reveals that presequences contain an abundance of polar, hydrophobic, and basic amino acids with a marked absence of acidic residues (von Heijne, 1986). Based on the regular spacing of basic residues within presequences, it is predicted that presequences do, however, share the ability to organize into amphiphilic structures in apolar environments such as mitochondrial membranes (von Heijne, 1986). This prediction was proven correct when interactions between synthetic presequence peptides and model membranes were studied (Roise *et al.*, 1986; Hoyt *et al.*, 1991). Further, importance of amphiphilicity in the import signal has been demonstrated by experiments in which artificial mitochondrial presequences, made from serine, leucine, and arginine, were found to direct import of passenger proteins fused to them if the presequences could form amphiphilic structures (Allison and Schatz, 1986; Roise *et al.*, 1988). Thus, structural requirements for a functional mitochondrial import signal appear rather modest. This, however, makes it difficult to understand how specificity of intracellular delivery is achieved.

An approach to this problem is to study synthetic presequences in both model and biological systems. Studies with the synthetic presequence of the F_1-ATPase[1] β-subunit precursor ($pF_1\beta$), the NH_2-terminal 20 amino acids, indicate this presequence can adopt an amphiphilic α-helical conformation in hydrophobic environments and can bind tightly to phospholipid monolayers. This presequence peptide, however, does not penetrate the hydrophobic core of the bilayers to disrupt the integrity of acidic phospholipid vesicles (Hoyt *et al.*, 1991). Interestingly, the $pF_1\beta$ presequence alone does not efficiently compete for *in vitro* import of full-length precursor ($K_i \cong 100$

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[1] The abbreviations used are: F_1-ATPase, soluble portion of the mitochondrial inner membrane bound ATPase complex; DiS-C3-5, 3′,3′-dipropylthiadicarbocyanine iodine. $\Delta\psi$, electrical potential across the inner mitochondrial membrane; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

μM, Hoyt *et al.*, 1991). However, studies with the preornithine carbamyltransferase presequence, amino acids 1-27, show that this peptide can completely block mitochondrial import at 5-10 μM, and collapse $\Delta\psi$ across the inner mitochondrial membrane unless excess reticulocyte lysate is present in the import reactions (Gillespie *et al.*, 1985). The presequence of cytochrome oxidase subunit IV (COX IV), amino acids 1-25, was found to disrupt artificial membranes as well as uncouple mitochondria (Roise *et al.*, 1986). On the other hand, shorter COX IV presequence peptides, retaining targeting information, were found to efficiently compete for import without apparently altering membrane integrity (Glaser and Cumsky, 1990).

Since fusion of the first 14 amino acids of $pF_1\beta$ to dihydrofolate reductase (DHFR) is sufficient to direct *in vitro* mitochondrial import of this normally cytosolic protein (Walker *et al.*, 1990), it is difficult to understand why the $F_1\beta$ presequence peptide does not efficiently compete for import. Several features of $pF_1\beta$ import, however, suggest why this result is observed. Like other precursors, $pF_1\beta$ can be translocated into mitochondria post-translationally in a sequential series of events which include binding to the outer mitochondrial membrane, interaction with outer membrane proteins (MOM 19, MAS 70, and ISP 42; Sollner *et al.*, 1989; Hines *et al.*, 1990; Vestweber *et al.*, 1989) and membrane potential ($\Delta\psi$)-dependent insertion through the inner membrane membrane (Gasser *et al.*, 1982; Schleyer *et al.*, 1982). Translocation is coupled to an ATP-dependent interaction with mitochondrial HSP 70 (Kang *et al.*, 1990) and is followed by cleavage of the presequence and Hsp60 assisted assembly into the F_1-ATPase complex in the mitochondrial matrix (Cheng *et al.*, 1989). However, $pF_1\beta$ import differs in that insertion of this precursor through the inner membrane requires cytosolic ATP hydrolysis in addition to $\Delta\psi$ (Chen and Douglas, 1987a; Pfanner *et al.* 1987; Eilers *et al.*, 1987). Import of other precursors, with the exception of the ADP/ATP carrier protein (Pfanner *et al.*, 1987), requires ATP but only in the matrix (Hwang and Schatz 1990; Miller and Cumsky, 1991). Cytosolic ATP consumed in import could be hydrolyzed by molecular chaperones during interactions with $pF_1\beta$ and possibly by other soluble or membrane-associated translocation factors (Ohta and Schatz 1984; Chen and Douglas, 1987b; Pfanner and Neupert, 1987; Deshaies *et al.*, 1988; Murakami *et al.*, 1988; Sheffield *et al.*, 1990; Murakami and Mori, 1990). Since factor-dependent ATP hydrolysis is partially responsible for insertion of the $pF_1\beta$ presequence into mitochondrial membranes, one explanation for the lack of import inhibition by the $pF_1\beta$ presequence peptide, $F_1\beta$1-20, may be that more than the presequence is required for high affinity interaction with soluble or membrane-bound import components required for insertion into mitochondrial membranes.

To test the relationship between length of presequence peptides and their ability to block mitochondrial import, we synthesized several $F_1\beta$ presequence peptides of varying length and tested their ability to compete for *in vitro* mitochondrial protein import. Herein we report that presequence peptides, of varying amphiphilicity, containing 14 amino acids in addition to the presequence, block import half-maximally at 25-1000-fold lower concentrations than a peptide containing the presequence alone. Import competition was deemed specific in that it was reversible and could be competed by full-length precursor protein. Additionally, presequence peptides, at concentrations which completely block import, had no apparent effect on binding of full-length precursors to mitochondria. Thus, import competition appears to occur at a point beyond the initial interaction of precursor proteins with mitochondria.

EXPERIMENTAL PROCEDURES

Strains and Media—The *Escherichia coli* strain MC1066 (F-Δ lac X74, galU galK rpsL hsdR trpC9830 leuB600 pyrF74::Tn5) (Casadaban and Cohen, 1980) grown in LB medium (Miller, 1972) supplemented with the sodium salt of ampicillin (100 μg/ml) was used to select transformants and amplify plasmids (Maniatias *et al.*, 1982). Mitochondria were isolated from the *Saccharomyces cerevisiae* strain D273-10B (Matα). Cells were grown in semisynthetic salts, 2% lactate medium (Daum *et al.*, 1982) to an optical density at 600 nm of 2-4 and then harvested. The doubling time of D273-10B in this media is approximately 2.5 h.

DNA Techniques—Transformation of *E. coli* strain MC1066 and preparation of small scale plasmid DNA were as described (Maniatis *et al.*, 1982). Restriction digestions were as described by the commercial suppliers. Linearized plasmid DNA for *in vitro* transcription was extracted two times with chloroform/phenol (1:1), one time with chloroform/isoamyl alcohol (24:1). DNA was then precipitated with 70% ethanol and isolated by centrifugation. The resultant pellet was rinsed with 70% ethanol, dried, and then resuspended in 10 mM Tris, pH 7.4, made 1 mM in EDTA at 1 μg/ml.

In Vitro Transcription and Translation—The genes coding for precursors to $F_1\beta$, the F_1-ATPase α-subunit ($pF_1\alpha$) and the ADP/ATP carrier protein (pAAC) were placed under control of the phage T_7 polymerase promoter by ligation into plasmid pT_7-2 (Promega) as described previously: pT_7ATP_2 (Chen and Douglas, 1987a), pT_7ATP_1 (Takeda *et al.*, 1986), and pT_7AAC_1 (Smagula and Douglas, 1988). The respective plasmids, pT_7ATP_2, pT_7ATP_1, and pT_7AAC_1 were linearized with *Hin*dIII, *Bam*HI, and *Eco*RI. Two to 5 μg of linearized plasmid DNA was added to transcription reactions containing T_7 polymerase (Promega Corp., Madison, WI) as described earlier (Chen and Douglas, 1987a). Products of transcription reactions were extracted two times with chloroform/phenol (1:1), one time with chloroform/isoamyl alcohol (24:1). mRNA was then precipitated by addition of sodium acetate (300 mM) and ethanol (70%) and isolated by centrifugation. The resultant pellet was rinsed with 70% ethanol, dried, and then resupended in 50 μl of RNase-free H_20 (Maniatias *et al.*, 1982). Purified mRNA transcripts (5 μl) were used immediately in translation reactions or were stored frozen at −80 °C. Biosynthesis of ^{35}S-labeled precursor protein from mRNA transcripts was carried out in nuclease-treated reticulocyte lysate exactly as instructed by the supplier (Promega Corp., Madison, WI) except ^{35}S-translabel (1059 Ci/mmol, ICN Biomedicals, Irvine CA) was substituted for [^{35}S]methionine. Translation reactions were used immediately in import reactions or were stored frozen at −80 °C.

In Vitro Import Experiments—Cells grown in lactate medium were harvested, and mitochondria were isolated as described (Gasser *et al.*, 1982) except after isolation they were resuspended to 5 mg/ml in 1.2 M Sorbitol made 1 mM in EDTA and 10 mM MOPS buffer, pH 7.4 (SEM), aliquoted into 250-μl fractions, and flash frozen in liquid N_2. *In vitro* import of ^{35}S-labeled precursor protein was carried out in 100-μl reaction mixtures containing 200 mM sucrose, 10 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 10 mM succinate, 25 mM creatine phosphate, 2.5 mg/ml of creatine phosphokinase, 3% BSA, and mitochondria (40-60 μg), thawed on ice just before use. Except where noted, mitochondria were preincubated on ice with or without peptide in import buffer for 5 min prior to start of the import reactions by addition of ^{35}S-labeled precursor and shift to 25 °C. Following incubation at 25 °C for 20 min, mitochondria were reisolated, resuspended in SEM, reisolated again and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) and fluorography (Chamberlin, 1979). Quantitation of fluorograghs was achieved using model 300A Laser densitometer (Molecular Dynamics, Sunnyvale, CA). Quantitation of x-ray film exposed to the same gel for various time periods confirmed linearity of the observed signal.

Synthetic $F_1\beta$ Peptides—Presequence peptides were synthesized by Dr. David Klapper of the Department of Microbiology at University of North Carolina and Immuno-Dynamics (La Jolla, CA). Following synthesis and hydrogen fluoride cleavage all peptides were HPLC purified on a C4-Vydac column. The mobile phase consisted of 0.1% trifluoroacetic acid and acetonitrile mixed 1:1. Column buffer was removed by freeze drying peak fractions, and peptides were stored in a vacuum desiccator until use. The sequence and amino acid composition of peak fractions was confirmed by standard techniques. Pep-

tide stock solutions (1 mM) were made by weighing out peptide and then dissolving it in 10 mM HEPES, ph 7.4. Typically, 2 ml of peptide stock solutions were made, aliquoted, and then freeze dried. Concentrations of peptide stock solutions were verified by quantitative amino acid analysis. Just before use, peptide solutions were reconstituted to their original volume with water. All peptides were freely soluble in water.

Measurement of Mitochondrial Membrane Potential—Qualitative changes in $\Delta\psi$ brought on by $F_1\beta$ presequence peptides were assayed by monitoring increases in fluorescence of the cationic fluorescent dye 3,3-dipropylthiadicarbocyanine (DiS-C3-5, Molecular Probes, Eugene, OR) after peptide addition reaction mixtures containing dye-loaded mitochondria. DiS-C3-5 binds mitochondria exhibiting $\Delta\psi$ with a resultant decrease in DiS-C3-5 fluorescence. Agents which reduce $\Delta\psi$ cause release of DiS-C3-5 from mitochondria which is exhibited by an increase in DiS-C3-5 fluorescence (Sims *et al.*, 1974). Mitochondria (0.60 mg/ml) were incubated in 3-ml reaction mixtures containing 200 mM sucrose, 10 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 10 mM succinate, 3% BSA, and 2.0 μM DiS-C3-5, added from an ethanolic stock (150 μM). Reaction mixtures were maintained at 20 °C and stirred continuously through out the incubation period. Changes in fluorescence were monitored with a Perkin-Elmer model MPF-3 Fluorometer equipped with a Hitachi-OPD 33 strip chart recorder. Excitation was set at 620 nm and emission at 680 nm. Under these conditions mitochondria maintained a membrane potential for at least 25 min.

RESULTS

Peptide Length Is Important for Import Competition—Previous results from *in vitro* competition studies with the synthetic peptide $F_1\beta$1–20 demonstrate that the $F_1\beta$ presequence alone does not efficiently compete for mitochondrial import ($K_i \cong 100$ μM). This could result from inadequate information or length within $F_1\beta$1–20 to allow for high affinity binding to import factors or failure of the peptide to insert deep enough into the outer membrane to gain access to import components. To test the length dependence of peptide inhibition of mitochondrial import, peptides consisting of the 19 amino acid presequence of $F_1\beta$ plus adjacent amino acids in the mature protein were synthesized and HPLC purified (see "Experimental Procedures"). Depicted in Fig. 1 is the wild type sequence for the first 51 amino acids of $pF_1\beta$ and the synthetic $F_1\beta$ peptides used in this study. Where noted, additional amino acids have been included to allow for iodination and chemical coupling of the peptides in other studies.

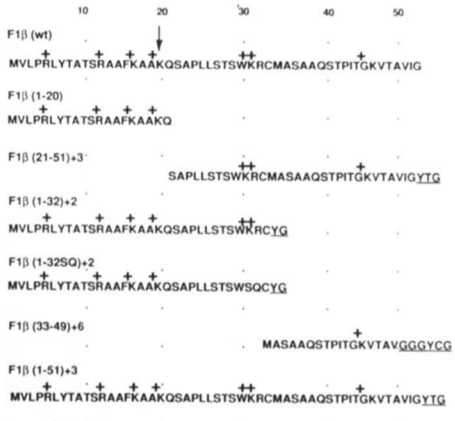


FIG. 1. **Amino acid sequences of synthetic $F_1\beta$ peptides used in this study.** The *arrow* between lysine 19 and glutamine 20 in the wild type $pF_1\beta$ sequence marks the presequence cleavage site. Regions of *underlined* sequence denote amino acids engineered into the peptides to allow for iodination of the peptides in other studies. All peptides were HPLC purified prior to use (see "Experimental Procedures").

Import competition by $F_1\beta$ peptides was tested in an *in vitro* import assay utilizing ^{35}S-labeled $F_1\beta$ precursor protein translated in reticulocyte lysate and mitochondria isolated from *S. cerevisiae* (see "Experimental Procedures"). This assay is based on quantitation of $\Delta\psi$-dependent processing of the precursor protein to the mature form which occurs only if the precursor is translocated into the mitochondrial matrix. Assays with $F_1\beta$1–20 (Fig. 2) and the control peptide $F_1\beta$33–49+6 (not shown) confirmed earlier results which demonstrated that neither peptide is an effective import inhibitor (Hoyt *et al.*, 1991). However $F_1\beta$1–32+2, which contains 14 amino acids in addition to the presequence, competed for import in a dose-dependent manner, and half-maximal inhibition occurred at around 0.12 μM (Fig. 2). Similar results were obtained with $F_1\beta$1–51+3 (Fig. 2). This K_i represents about a 1000-fold increase in sensitivity of import to presequence containing peptide when compared to $F_1\beta$1–20 and is in the range of those observed for import competition by purified full-length precursor proteins (Pfaller *et al.*, 1988; Sheffield *et al.*, 1990). Others have shown that import information in addition to the presequence is present within the first 34 amino acids of $preF_1\beta$ (Bedwell *et al.*, 1987) and thus,

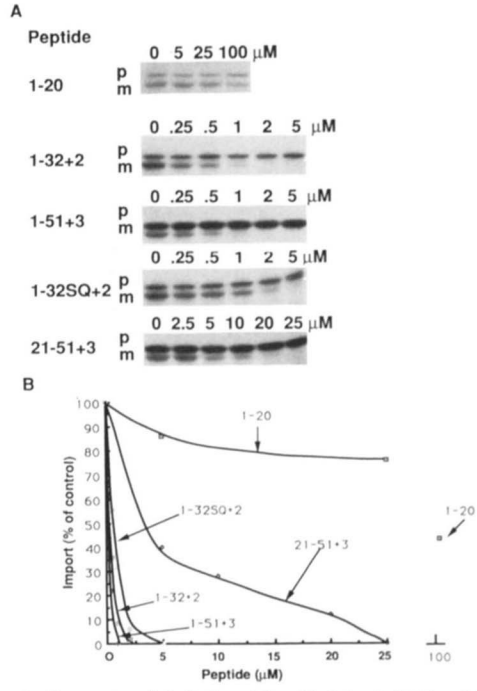


FIG. 2. **Import of full-length $pF_1\beta$ is inhibited by $F_1\beta$ presequence peptides.** *A*, isolated yeast mitochondria (600 μg/ml) were incubated in reactions mixtures containing 200 mM sucrose, 10 mM HEPES, pH 7.4, 3% BSA, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 10 mM succinate, 25 mM creatine phosphate, 2.5 mg/ml creatine phosphokinase, ^{35}S-labeled precursor translated in reticulocyte lysate, and $F_1\beta$ presequence peptide as indicated in a final volume of 100 μl. Mitochondria were preincubated on ice in import buffer, with and without peptide, 5 min prior to start of import reactions by addition of ^{35}S-labeled precursor and shift to 25 °C. Following incubation for 20 min, mitochondria were reisolated, washed, and analyzed for import as described under "Experimental Procedures." *B*, fluorographed gels were quantitated for mature $F_1\beta$ formed during import reactions by laser densitometry. Values are expressed as % of mature protein formed in the absence of peptide, set arbitrarily at 100%, and are plotted *versus* the concentration of respective peptide. It should be noted that in the presence of $F_1\beta$ peptides none of the precursor protein found associated with mitochondria was protected from digestion by proteinase K (not shown), indicating that $F_1\beta$ presequence peptides inhibit import and not processing of the precursor to the mature form under these experimental conditions.

this may be the cause for increased sensitivity of import to the longer $F_1\beta$ peptides. To address this possibility two mutant peptides of identical length to $F_1\beta$1–32+2 were synthesized, $F_1\beta$1–32SQ+2 and 21–51+3 (Fig. 1). $F_1\beta$1–32SQ+2 contained conservative replacements of the lysine and arginine at positions 30 and 31 with serine and glutamine, respectively. These basic residues are predicted to extend the amphiphilic structure of the presequence and thus strengthen the import signal (Bedwell *et al.*, 1987). $F_1\beta$21–51+3 lacks the presequence portion of $pF_1\beta$ but still retains some predicted ability to form an amphiphilic helix (see under "Discussion"). When tested in competition assays, a 4-fold higher concentration of $F_1\beta$1–32SQ+2 was required to half-maximally block import of full-length precursor ($K_i \cong 0.5$ μM) when compared to $F_1\beta$1–32+2 (Fig. 2). However, 35 times more $F_1\beta$21–51+3 was required to compete for import than $F_1\beta$1–32+2 (Fig. 2). It is noteworthy that $F_1\beta$21–51+3 ($K_i \cong 4$ μM) remained 25 times more potent as an import competitor than $F_1\beta$1–20 ($K_i \cong 100$ μM, Fig. 2). Hence, additional length appears important, perhaps more important, than the ability to form an extended amphiphilic helix in conferring ability of synthetic $F_1\beta$ peptides to compete for mitochondrial protein import.

Peptides Compete for Mitochondrial Import in a Reversible Manner—Some, but not all, amphiphilic peptides interact with and disrupt lipid bilayers (Segrest *et al.*, 1990). Disruption of the inner mitochondrial membrane and resultant dissipation of the electrochemical gradient required to drive protein translocation into mitochondria has been associated with inhibition of mitochondrial import by a few synthetic presequence peptides (Gillespie *et al.*, 1985; Roise *et al.*, 1986; Glaser and Cumsky, 1990). To examine the possibility that $F_1\beta$ peptides were acting in a similar manner, dose-dependent inhibition of mitochondrial import and changes in mitochondrial $\Delta\psi$, assayed with the cationic fluorescent dye DiS-C3–5, were compared under conditions used to assay *in vitro* mitochondrial import (see "Experimental Procedures"). Presented in Fig. 3, *A-E*, are curves representing increases in fluorescence, corresponding to decreases in $\Delta\psi$, observed upon addition of respective $F_1\beta$ peptides to reaction mixtures containing DiS-C3–5-loaded mitochondria. These values are expressed in percent of the maximal increase in fluorescence observed upon addition of the K^+ ionophore valinomycin (2.5 μg/ml) to reaction mixtures. Also shown is the corresponding effect of respective $F_1\beta$ peptides on import of $pF_1\beta$ assayed under similar conditions. In most cases, $F_1\beta$ peptides dissipated mitochondrial $\Delta\psi$ to about 80% of maximal values observed with valinomycin at concentrations below 5 μM (Fig. 3, *C-E*). $F_1\beta$1–20 and $F_1\beta$21–51+3 are the exceptions (Fig. 3, *B* and *F*).

Dissipation of $\Delta\psi$ by $F_1\beta$ peptides made it difficult to resolve the extent of import inhibition due to competition from that resulting from reduced membrane potential. However, in all cases import inhibition by $F_1\beta$ peptides exceeded the effect on $\Delta\psi$ (Fig. 3, *B-F*). This is in contrast to the effect observed with valinomycin plus K^+ which simply dissipates $\Delta\psi$ across the mitochondrial membrane (compare Fig. 3, *A* to *B-F*). The peptides $F_1\beta$ 1–32+2 and 21–51+3 blocked import 50 and 100% at peptide concentrations which dissipate $\Delta\psi$ less than 5 and 25%, respectively (Fig. 3, *C* and *F*). Titration experiments with valinomycin (Fig. 3*A*) demonstrate, however, that when mitochondrial $\Delta\psi$ is reduced 25% import is inhibited only 25%. Thus, at least 75% of maximal import inhibition observed in the presence of $F_1\beta$1–32+2 and $F_1\beta$21–51+3 is likely to result from competition for a specific import step.

The data in Fig. 3 indicate that some but not all import competition noted with the longer $F_1\beta$ peptides might be due

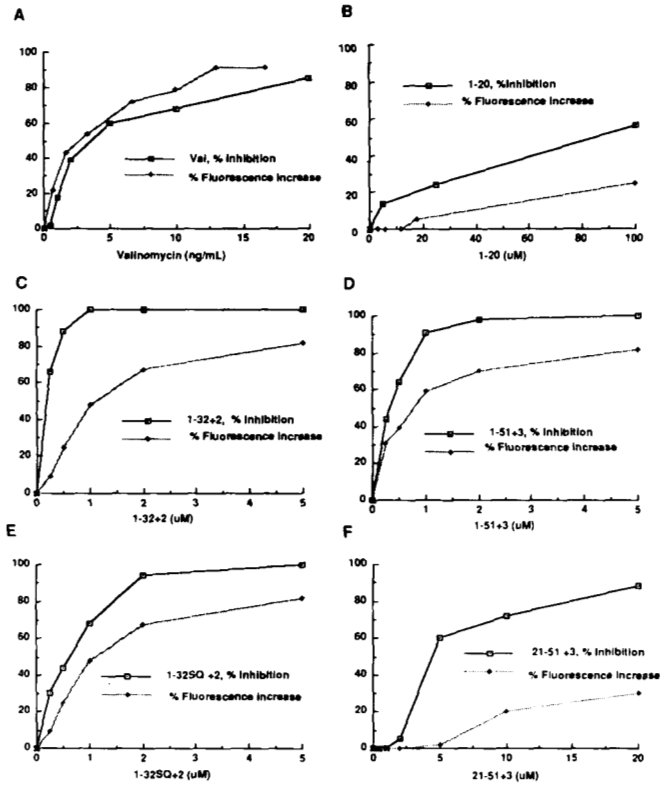


FIG. 3. **Inhibition of mitochondrial import by $F_1\beta$ presequence peptides exceeds dissipation of mitochondrial $\Delta\epsilon$.** For measurement of $\Delta\epsilon$, isolated mitochondria (600 μg/ml) were incubated in 3 ml of reaction mixtures containing 200 mM sucrose, 10 mM HEPES, pH 7.4, 3% BSA, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 10 mM succinate, and 2.0 mM DiS-C3–5 (see "Experimental Procedures"). Reactions were stirred continuously and maintained at 20 °C. Presequence peptide or valinomycin-dependent dissipation of $\Delta\psi$, represented as an increase in fluorescence intensity, are plotted as a percentage of the maximal increase in fluorescence intensity observed when valinomycin (2.0 μg/ml) was added to reaction mixtures (*% fluorescence increase*), arbitrarily set at 100%. Each curve represents the change in fluorescence intensity observed upon titration of increasing quantities of respective $F_1\beta$ peptide or valinomycin into a single reaction mixture. Also shown is the corresponding effect of respective $F_1\beta$ peptide or valinomycin on import of full-length $pF_1\beta$ assayed in companion import reactions. Values (*% inhibition*) are expressed as percent inhibition of mature $F_1\beta$ formation as compared mature $F_1\beta$ formed in import reactions in the absence of peptide which was arbitrarily set at 0% in each case.

to dissipation of the threshold $\Delta\psi$ required to maintain import competency of mitochondria. It was reasoned that if membrane potential is necessary for import, the movement of competitor peptides through the import apparatus should compete for a portion of $\Delta\psi$ required for efficient import. If this were so two conditions should be demonstrable to document the specificity of import inhibition by $F_1\beta$ peptides. The inhibition by peptides should be reversible and the inhibition should be competed by full-length precursor. To test the first condition we asked if inhibition of import by $F_1\beta$1–51+3 was reversible. Mitochondria were first incubated with 0 or 1.5 μM peptide, a concentration which dissipates mitochondrial $\Delta\psi$ about 65% (Fig. 3*D*), and ^{35}S-labeled $F_1\beta$ precursor in reticulocyte. After initial incubation, mitochondria from respective reaction mixtures were reisolated, washed, resuspended in import buffer, and divided into three equal parts. One-third was assayed directly for import in the first incubation, another was reincubated with untranslated reticulocyte lysate in the absence of peptide, and the final third was reincubated with additional ^{35}S-labeled precursor in reticulocyte lysate in the

absence of peptide. In the primary incubation, $F_1\beta$1–51 reduced import to about 10% of control levels (Fig. 4). However, import competence was restored to control levels after wash and reincubation of peptide treated mitochondria (Fig. 4). Control experiments with valinomycin indicate that maturation of precursor in the second incubation is dependent upon $\Delta\psi$ and thus represents precursor imported into the mitochondrial matrix (not shown). Hence, these data demonstrate that import inhibition by $F_1\beta$ peptides is transient and does not result in permanent damage to mitochondria.

In a second experiment we asked if import inhibition by $F_1\beta$ peptide could be overcome by increasing the amount of full-length precursor protein present in import reactions. This was accomplished by incubating mitochondria in separate reaction mixtures with 1 and 30 μl of reticulocyte lysate containing ^{35}S-labeled $F_1\beta$- or $F_1\alpha$-subunit precursors. Twenty-nine μl of untranslated reticulocyte was added to reaction mixtures containing 1 μl of programed lysate to make all reactions chemically equivalent. The presence of $F_1\beta$1–51+3 reduced import of $pF_1\alpha$ and $pF_1\beta$, about 90 and 75%, respectively, when 1 μl of labeled precursor was present in import reactions (Fig. 5, *B* and *C*). However, when 30 μl of $pF_1\alpha$ was present in a parallel import reaction, import was inhibited only 50% by $F_1\beta$1–51+3 (Fig. 5, *E* and *F*). $pF_1\beta$ was less effective at competing with $F_1\beta$ peptide for import inhibition (Fig. 5, *E* and *F*), this may indicate that the import apparatus has a higher affinity for $pF_1\alpha$ than $pF_1\beta$. Both $pF_1\alpha$ and $pF_1\beta$ were imported at higher efficiencies when 30 times precursor was present in import reactions (Fig. 5, *B* and *E*). It is not clear why this result was observed. It could be that increased import efficiency results from a greater percentage of precursor being available for import at high precursor concentration because a lower percentage of total precursor is tied up in nonspecific interactions during import reactions.

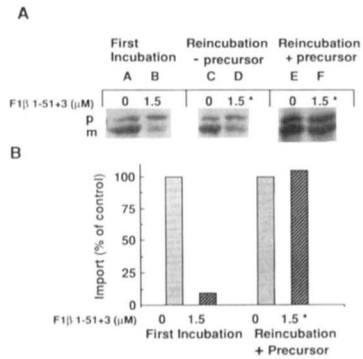


FIG. 4. **Inhibition of import by $F_1\beta$1–51+3 is reversible.** *A*, mitochondria (600 μg/ml) were incubated with reticulocyte lysate containing ^{35}S-labeled $pF_1\beta$ in 300 μl of reaction mixtures as described in the legend to Fig. 2. After initial incubation for 20 min at 25 °C, reactions were divided into three equal parts, mitochondria were reisolated from each, and then resuspended in import buffer and either analyzed directly for import in the first incubation or reincubated in 100 μl of import reactions at 25 °C for 25 min. One aliquot of mitochondria from each initial import condition (no addition and + peptide) was reincubated, in the absence of $F_1\beta$ peptide, with untranslated reticulocyte lysate or lysate containing ^{35}S-labeled precursor. * denotes the peptide concentration mitochondria were treated with in the first incubation. After the second incubation mitochondria were reisolated and analyzed for import as described under "Experimental Procedures." *B*, quantitation of bands corresponding to the mature protein in the fluorographed gel. To calculate import of $pF_1\beta$ into mitochondria in the second incubation, mature protein present in mitochondria resultant from the initial import reaction was subtracted from mature protein present in mitochondria after reincubation with ^{35}S-labeled $pF_1\beta$. (*E–C* and *F–D* = import in the second reaction).

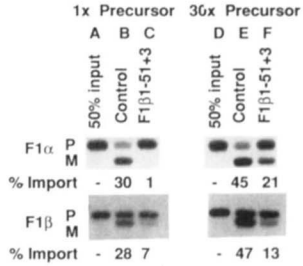


FIG. 5. **Import inhibition by $F_1\beta$1–51+3 is competed by full-length precursor.** Import reactions contained 1 or 30 μl of reticulocyte-translated ^{35}S-labeled $pF_1\alpha$ or $pF_1\beta$ and were carried out as described in the legend to Fig. 2. All reactions contained a total of 30 μl of reticulocyte lysate. % of import represents the percentage of precursor present in respective reaction mixtures which was converted into the mature form. *1×* represents 7.3 and 8.0 × 10^3 cpm of $F_1\alpha$ and $F_1\beta$ precursor, respectively. Quantitation of gels was accomplished by scanning the fluorographed gels with a densitometer. When present $F_1\beta$1–51+3 was 1.5 μM. In *lanes A* and *D*, 50% input, half of a complete import reaction in which BSA was omitted and poisoned with 2.0 μg/ml of valinomycin was loaded onto the gel.

Irrespective of this, from these data it is clear that mitochondria remain import competent in the presence of $F_1\beta$ peptides and that import inhibition is likely to result from competition between full-length precursor and peptide for specific import step(s).

Since $F_1\beta$1–51 inhibited import of two presequence containing peptides we next asked what effect peptide had on import of a precursor which lacked a presequence. The adenine nucleotide transporter (pAAC), which contains several internal targeting domains, was used for this purpose. Import of pAAC unlike $pF_1\beta$ does not require a membrane potential across the mitochondrial inner membrane to deliver the precursor to a protease protected location. However, membrane insertion of pAAC into the inner membrane does require $\Delta\psi$ across the inner membrane (Pfanner *et al.*, 1987). In earlier studies, the delivery of pAAC to its transmembrane location in the inner membrane was readily determined by defining the extent to which the protease protected form becomes resistant to extraction by sodium carbonate at pH 11.5 (Pfanner *et al.*, 1987; Smagula and Douglas, 1988). After the simultaneous import of the $pF_1\alpha$ and the pAAC protein, the proteins associated with mitochondria and protected from external protease were respectively characterized for their extractability by sodium carbonate. Shown in the center section of Fig. 6 (no additions), are three lanes displaying total imported protein and the protein of the total remaining in the supernatant or within the pellet following extraction with sodium carbonate, respectively. In the absence of peptide, 50% of input $pF_1\alpha$ was imported to a protease protected space. Addition of $F_1\beta$1–51+3 blocked entry of $pF_1\alpha$ to a protected space by about 95% (Fig. 6). In contrast, import of pAAC is reduced only about 20%. Quantitation of the total imported AAC protein which became resistant to carbonate extraction revealed that the presence of $F_1\beta$1–51+3 had no effect on the energetics required for membrane insertion. Fig. 6 shows that slightly over 50% of the total protected pAAC is transmembrane, independent of the presence of the inhibitory peptide. In contrast, in the absence of membrane potential (+ valinomycin), the amount of pAAC resistant to carbonate extraction was reduced to 16% of the total protected protein. Import of $pF_1\alpha$ was not detected in the absence of membrane potential. It should be noted that at 10 μM $F_1\beta$1–51+3 import of pAAC to a non-carbonate extractable space was inhibited by about 75% (not shown). These results indicate that mitochondria remain import competent for pAAC in the presence of $F_1\beta$1–

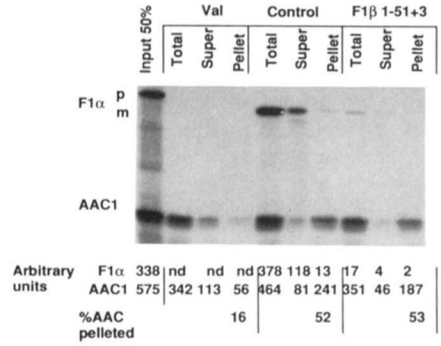


FIG. 6. **Import of F_1a but not AAC2 is sensitive to $F_1\beta$1–51+3 in the same mitochondria.** A mixture of reticulocyte containing ^{35}S-labeled $pF_1\alpha$ and pAAC was incubated with mitochondria for 20 min at 25 °C as described in the legend to Fig. 2. After incubation, mitochondria were reisolated, and resuspended in 100 μl of SEM buffer containing 100 μg/ml proteinase K and incubated for 30 min on ice. Phenylmethylsulfonyl fluoride was then added to 1 mM and reaction mixtures were split into 2 aliquots. One aliquot was made 0.1 M in sodium carbonate, pH 11.5, and incubated on ice for 30 min while the other was stored on ice until analysis for import. Following incubation with sodium carbonate, soluble proteins were separated for integral membrane proteins by centrifugation in a Beckman airfuge for 30 min at 30 psi, and both the pellet and supernatants were analyzed for import as described under "Experimental Procedures."

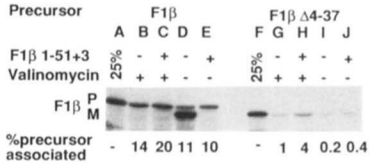


FIG. 7. **Binding of the $F_1\beta$ subunit precursor to mitochondria is presequence dependent, independent of $\Delta\epsilon$, and insensitive to $F_1\beta$ peptide.** Mitochondria (500 μg/ml) were incubated with ^{35}S-labeled $pF_1\beta$ or the mutant $F_1\beta\Delta$4–37, which is missing amino acids 4–37, for 20 min at 25 °C as described in the legend to Fig. 2. When added $F_1\beta$1–51+3 and valinomycin were 1.5 and 2.0 μM, respectively. Precursor protein associated with washed mitochondrial pellets was quantitated by scanning the fluorographed gel with a densitometer.

51+3 at concentrations which completely block import of presequence containing precursors. Further, these data infer that pAAC and presequence-containing precursors have different affinities for the import apparatus and may require $\Delta\psi$ to different extents to drive insertion into the inner membrane.

$F_1\beta$ Peptides Block Import at a Step beyond Binding to Mitochondria—$F_1\beta$ presequence peptides completely block import without diminishing binding of $pF_1\beta$ to mitochondria (Fig. 2). Additionally, inhibition of $pF_1\alpha$ import by $F_1\beta$ presequence peptides is accompanied by a large accumulation of precursor protein on the mitochondrial surface which is not observed in the absence of peptide (Fig. 5, *B*, *C* and *E*, *F*). These results are of interest since binding of precursor proteins to the outer mitochondrial membrane is proposed to involve specific interaction of presequences with trypsin-sensitive import receptors (Pfanner *et al.*, 1991). To examine $F_1\beta$ precursor binding to mitochondria in greater detail, we tested presequence dependence and trypsin sensitivity of binding as criteria for specificity. We compared the binding the $pF_1\beta$ mutant $F_1\beta\Delta$4–37, which is missing amino acids 4–37, to that of wild type $pF_1\beta$ (Fig. 7). In deenergized mitochondria (+ valinomycin) 14 and 20% of input $pF_1\beta$ bound to mitochondria in the absence and presence $F_1\beta$1–51+3, respectively (Fig. 7, *B* and *C*). When import by energized mitochon-

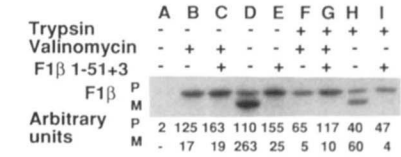


FIG. 8. **$F_1\beta$ peptide blocks import but not binding of $pF_1\beta$ to trypsin-treated mitochondria.** ^{35}S-Labeled $pF_1\beta$ was incubated with either mock or trypsin-treated mitochondria at 25 °C for 20 min as described in the legend to Fig. 2. Mock treated mitochondria were incubated on ice for 30 min with trypsin (0.1 mg/mg of mitochondria) which was previously inactivated with a 2-fold weight excess of bovine trypsin inhibitor. Trypsin-treated mitochondria were incubated as above except bovine trypsin inhibitor was added after incubation. Mitochondria were then reisolated and resuspended to the original concentration of 5 mg/ml in SEM and used for import assays. When present, valinomycin and $F_1\beta$1–51+3 were 2.0 and 1.5 μM, respectively. *Lane A* represents binding observed in the absence of mitochondria in import reactions. Bands on the fluorographed gels corresponding to precursor and mature protein were quantitated by laser densitometry and are expressed in arbitrary units.

dria was completely blocked by $F_1\beta$1–51+3, the amounts of precursor bound to mitochondria were the same as those bound in the absence of peptide (Fig. 7, *D* and *E*). Binding $F_1\beta\Delta$4–37 to mitochondria was 14- and 50-fold lower than that observed with $pF_1\beta$, under energized and deenergized conditions, respectively (*B versus G* and *D versus I*). Thus, results with $F_1\beta\Delta$4–37 show that although binding of precursor protein to mitochondria is not competed by presequence peptide, binding is presequence-dependent.

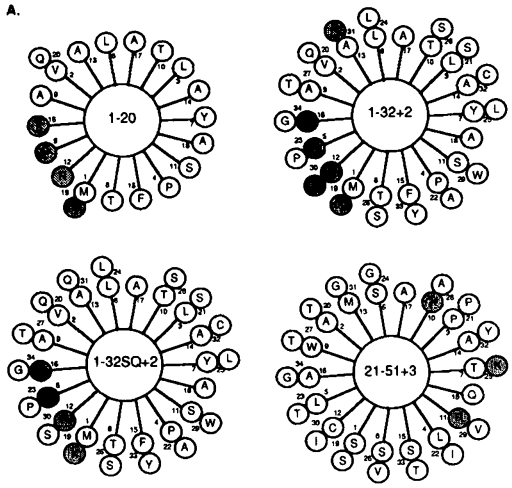
The role of import receptors in mediating the observed mitochondrial binding of $pF_1\beta$ was addressed by comparison of $pF_1\beta$ binding to control mitochondria and to mitochondria digested with trypsin at concentrations which reduce import but do not disrupt mitochondrial integrity (not shown). Trypsin treatment of mitochondria reduced import 78% (Fig. 8, *D* and *H*), but residual import remained sensitive to peptide (Fig. 8, *H* and *I*). Consistent with $pF_1\beta$ binding to high affinity sites on the outer membrane, binding of $pF_1\beta$ to mitochondria was reduced about 50–70% by trypsin treatment as compared to mock treated controls (Fig. 8, *B versus F*, *E versus I*, and *D versus H*). Binding of $pF_1\beta$ to trypsin-treated mitochondria was insensitive to $F_1\beta$ peptide (Fig. 8, *H versus I*). Although trypsin digestion reduced $pF_1\beta$ binding to mitochondria, it appears bound precursor is imported with the same efficiency observed with mock treated mitochondria. This is indicated by the observation that the ratio of precursor to mature protein found associated with both control and digested mitochondria is constant (Fig. 8, *D* and *H*). At present it is not clear whether residual binding and import is mediated by outer membrane import proteins not digested by trypsin or by a bypass route (Pfaller *et al.*, 1988). Nonetheless, $pF_1\beta$ binding to mitochondria is 50–70% dependent on trypsin-sensitive elements, is independent of $\Delta\psi$, and presequence-dependent. However, we observe that binding to the mitochondrial surface is insensitive to $F_1\beta$ presequence peptide. Thus, we conclude that import competition by $F_1\beta$ peptides occurs at a site beyond initial binding of precursor proteins to mitochondria.

DISCUSSION

The role performed by mitochondrial presequences in orchestrating specific protein delivery and translocation events within the cell remains obscure. One of several key questions which remains is how these sequences which share only the potential to form an amphiphilic structure mediate specific binding to mitochondria. In the present study we have examined presequence-dependent binding of the $F_1\beta$-subunit

precursor to isolated mitochondria and the ability of synthetic peptides composed of the NH_2-terminal residues of $pF_1\beta$ to compete for this binding. We find, quite unexpectedly, that presequence dependent binding to mitochondria is not competed for in any detectable manner by $F_1\beta$ presequence peptides. However, efficient competition for import of the bound precursor can occur at a point beyond binding to receptor components on the organelle surface.

Earlier studies demonstrated that although the $F_1\beta$ presequence peptide, $F_1\beta 1$–20, binds with high affinity to phospholipid vesicles, $K_a = 50 \times 10^{-8}$ M, this peptide is a weak import competitor, $K_i \cong 100 \times 10^{-6}$ M (Fig. 2, Hoyt *et al.*, 1991). In present study we find that three different $F_1\beta$ peptides, all 34 amino acids in length, $F_1\beta 1$–32+2, $F_1\beta 1$–32 SQ+2, and $F_1\beta 21$–51+3 compete for import half-maximally at 1000-, 250-, and 25-fold lower concentrations, respectively, than $F_1\beta 1$–20, (Fig. 9*B*). Qualitative and quantitative predictors of peptide amphiphilic character, Shiffer-Edmundson helical wheel plots and the algorithm of Segrest (Shiffer and Edmundson, 1967; Segrest *et al.*, 1990), respectively, indicate $F_1\beta 1$–20 has the highest predicted hydrophobic moment/residue and charge density on its polar face of all peptides used in this study (Fig. 9, *A* and *B*). Thus, ability to form an amphiphilic α-helical structure with high charge density appears less important than overall length in conferring ability of $F_1\beta$ presequence peptides to inhibit mitochondrial import. This is supported by the observation that $F_1\beta 21$–51+3 has a calculated hydrophobic moment/residue which is 50% that of $F_1\beta 1$–20, but is 25 times more potent as an import competitor (Fig. 9*B*). On the other hand, $F_1\beta 1$–32+2 has about 30% more amphiphilic character, exhibits a higher charge density, and is 33 times more potent as an import competitor than $F_1\beta 21$–51+3 (Fig. 9, *A* and *B*). Based on the above observations we propose that both minimal length and minimal amphiphilic character are required for functional import signals. In the case of the $F_1\beta$-subunit precursor, the amphiphilicity of the presequence appears sufficient to direct import but the minimal length necessary for function requires additional residues. This would explain the observation that 14 amino acids of the $F_1\beta$ presequence can direct *in vitro* import of DHFR (Walker *et al.*, 1990) but the synthetic 20-residue $F_1\beta$ presequence peptide fails to efficiently compete for import (Fig. 2; see also Hoyt *et al.*, 1991).



B.

Peptide	1-20	1-32+2	1-32SQ +2	21-51+3
Ki (μM)	100	0.12	0.5	4.0
Residues	20	34	34	34
Mean hydrophobic moment per residue	0.41	0.33	0.27	0.23
Mean hydrophobicity per residue of nonpolar face	0.55	0.58	0.57	0.60

FIG. 9. **Amphiphilic and inhibitory properties of $F_1\beta$ competitor peptides.** *A*, helical wheel projections of $F_1\beta$ peptides (Shiffer and Edmundson, 1967). *Shaded circles* represent basic amino acids. *B*, tabulation of the inhibition constants and calculated values for the mean hydrophobic moment/residue and mean hydrophobicity/residue of non-polar face. Inhibition constants were determined from data presented in Fig. 2. The other values were calculated using an algorithm designed to predict the amphiphillic character of proteins (Segrest *et al.*, 1990).

Import competition by $F_1\beta 1$–51+3 and other synthetic presequence peptides (Gillespie *et al.*, 1985; Glaser and Cumsky, 1990) appears to take place within the contact site very near or at the membrane potential-dependent step. We propose that the peptide itself competes for energy-dependent transport of the full-length precursor in the contact site. This model would propose that when inhibitory peptide concentration is sufficient to saturate the $\Delta\psi$-dependent translocation device, import of peptide would consume a large portion of $\Delta\psi$ necessary to drive transport of other proteins. Upon completion of transport or removal of excess peptide, membrane potentials would reestablish for further energy-dependent activities. Several observations in the present study support this view. First, $F_1\beta 1$–20 binds avidly to membranes but is not able to penetrate deeply into phospholipid bilayers and disrupt phospholipid vesicles (Hoyt *et al.*, 1991). Second, although dissipation of mitochondrial $\Delta\psi$ was observed in the presence of longer $F_1\beta$ peptides less than 25% of maximal import inhibition can be attributed to this mechanism when import inhibition by $F_1\beta 1$–32+2 and $F_1\beta 21$–51+3 is compared to that by the uncoupler valinomycin (Fig. 3, *A* and *C*). Third, unlike valinomycin-treated mitochondria, if peptide-treated mitochondria are washed and reincubated in the presence of additional precursor, efficient uptake of newly added precursor is observed (Fig. 4). Fourth, in the presence of $F_1\beta$ peptide at concentrations sufficient to completely block import of $pF1\alpha$, $\Delta\psi$-dependent import of pAAC into the mitochondrial inner membrane is not affected. Fifth, peptide-dependent inhibition of import is reduced by addition of a 30-fold more full-length precursor to import reactions (Fig. 5). The inclusion of additional substrate for energy-dependent transport would not be expected to overcome inhibition by peptide if the full-length substrate and inhibitory peptide were acting at different places in the membrane. Thus, this result confirms that the two forms of the precursor directly compete in some manner at a specific import step

We also observe that peptide consisting of the import signal failed to inhibit binding of lysate translated full-length precursors to mitochondria (Figs. 2, 5, 7, and 8). These observations suggest that recognition events on the mitochondrial surface which may require outer membrane proteins (Sollner *et al.*, 1989, 1990; Hines *et al.*, 1990) may involve more than the presequence. Support for this conclusion is provided by the following observations. First, when import of $F_1\alpha$ was blocked by peptide $F_1\beta 1$–51+3 a significantly larger amount of precursor was found associated with mitochondria than in the absence of peptide (Fig. 5, *B*, *C*, *E*, and *F*). Similarly, at concentrations of $F_1\beta$ peptide which completely block import of $pF_1\beta$ no decrease in the binding of $F_1\beta$ precursor to mitochondria is observed (Fig. 7). This association of $pF_1\beta$ with mitochondria is dependent on the presequence (Fig. 7), independent of a membrane potential across the inner membrane (Fig. 7) and greatly enhanced by trypsin-sensitive elements on the mitochondrial surface (Fig. 8). This mitochondrial association of precursor on the surface appears (Figs. 7 and

8) as it has in earlier studies to be on the import pathway (Reizman *et al.*, 1983). Following a first incubation in the presence of an inhibitory concentration of $F_1\beta$1–51+3, 30–40% of the bound but non-imported $F_1\beta$ precursor is imported following removal of peptide and reincubation of mitochondria (not shown).

In comparing results presented here with those of previous studies with synthetic presequence peptides, we find that our results are in close agreement with observations made in import competition studies with synthetic truncated forms of the Cox IV presequence which still retain targeting information. (Glaser and Cumsky, 1990). As in our experiments, dissipation of mitochondrial $\Delta\psi$ by Cox IV presequence peptides was not responsible for import inhibition, and a competitive mechanism for import inhibition was indicated (Glaser and Cumsky, 1990). Additionally, import inhibition by Cox IV presequence peptides was not associated with a reduction in binding of precursor proteins to mitochondrial membranes (Glaser and Cumsky, 1990). Further experiments showed that Cox IV presequence peptide blocks import of pAAC at a site beyond translocation into the outer mitochondrial membrane but before insertion into the inner mitochondrial membrane (Glaser and Cumsky, 1990). $F_1\beta$ presequence peptides were found to block pAAC import at a similar site.[2] Thus, results from import competition studies with pAAC support interpretations from binding experiments that specific inhibition of mitochondrial import by synthetic presequence peptides occurs at a site beyond initial interaction of precursor proteins with mitochondria.

The present data are consistent with a model in which the presquence would serve to organize soluble factor(s) onto the precursor either co- or post-translationally. The factor(s) in this complex would maintain the precursor in a transport competent state and assist in targeting the precursor to the mitochondrial surface. It is quite possible that import receptors recognize both structural features of the precursor and soluble factor(s) to mediate specific binding. Such a model was recently proposed for the role of secB in facilitating binding of precursor proteins to the export apparatus in *E. coli.* (Hartl *et al.*, 1990).

Failure of synthetic $F_1\beta$ peptides to block binding of precursor proteins to mitochondria indicates they do not present a conformation or organization which promotes the binding of factors necessary for high affinity binding to import receptors. Instead they probably gain access to membrane-bound import components directly. This is indicated by ability of $F_1\beta$ peptides to compete for residual import in trypsin treated mitochondria (Fig. 8, *H* and *I*). The ability of mitochondrial precursors to be imported independent of the import receptor system is not without precedent. Presequence dependent import of cytochrome oxidase subunit Va (COX Va) precursor occurs at 100% of control rates in mitochondria treated with trypsin at concentrations which abolish import of other precursors (Miller and Cumsky, 1991). $F_1\beta$ peptides also compete for import of COX Va demonstrating they share a common import step (not shown). Interestingly COX Va import lacks or requires very low levels of ATP hydrolysis in the cytosol (Miller and Cumsky, 1991) demonstrating a relationship between bypass of trypsin-sensitive import factors and lack of a cytosolic ATP requirement for import of an authentic mitochondrial precursor. The above data suggest that proteinaceous import factors on the mitochondrial surface serve to recognize and discharge factors associated with precursor proteins which require factors for import and that some precursor proteins which are naturally import competent may not require the assistance of ATP-dependent translocation factors or proteinaceous import receptors.

[2] D. M. Cyr and M. G. Douglas, unpublished observations.

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