Mitochondrial Import of the ADP/ATP Carrier Protein in Saccharomyces cerevisiae

SEQUENCES REQUIRED FOR RECEPTOR BINDING AND MEMBRANE TRANSLOCATION*

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The ADP/ATP carrier of yeast (309 amino acids) is an abundant transmembrane protein of the mitochondrial inner membrane whose import involves well-defined steps (Pfanner, N., and Neupert, W. (1987) J. Biol. Chem. 262, 7528–7536). Analysis of the in vitro import of gene fusion products containing ADP/ATP carrier (AAC) sequences at the amino terminus and mouse dihydrofolate reductase (DHFR) at the carboxyl terminus indicates that the first 72 amino acids of the soluble carrier protein, a hydrophilic region of the protein, are not by themselves sufficient for initial binding to the AAC receptor on the mitochondrial surface. However, an AAC-DHFR gene fusion containing the first 111 residues of the ADP/ATP carrier protein exhibited binding to mitochondria at low temperature (2°C) and internalization at 25°C to a mitochondrial space protected from proteinase K in the same manner as the wild-type ADP/ATP carrier protein. The AAC-DHFR protein, in contrast to the wild-type AAC protein imported into mitochondria under optimal conditions, remained extractable at alkaline pH and appeared to be blocked at an intermediate step in the AAC import pathway. Based on its extraction properties, this AAC-DHFR hybrid is proposed to be associated with a proteinaceous component of the import apparatus within mitochondria. These data indicate that the import determinants for the AAC protein are not located at an extreme amino terminus and that protein determinants distal to the first 111 residues of the carrier may be necessary to move the protein beyond the alkaline-extractable step in the biogenesis of a functional AAC protein.

Transport of adenine nucleotides between mitochondrial and cytoplasmic compartments of the cell is mediated by an integral homodimeric protein in the mitochondrial inner membrane called the adenine nucleotide translocator or ADP/ATP carrier (Klingenber, 1981). The transport properties of the ADP/ATP carrier (AAC) have been examined in some detail using specific inhibitors of the translocator which are able to interact specifically with the protein from either the mitochondrial matrix or cytoplasmic face of the transmembrane structure. Analysis of the primary sequence of the translocator from a variety of sources indicates that the primary structure of this membrane-spanning transporter is highly conserved (Adrian et al., 1986). Recent analysis of different solute transporters of the mitochondrial inner membrane indicate that the ADP/ATP carrier is representative of a family of integral proteins in this membrane which share considerable sequence homology and membrane topology (Aquila et al., 1985; Runswick et al., 1987). It is possible that these structurally related proteins may share a common route of import and assembly in the inner membrane.

The ADP/ATP carrier protein is the most abundant protein in mitochondria from different sources, constituting 14–20% of the total mitochondrial membrane protein (Klingenber, 1986). Its biogenesis has been examined in detail using in vivo and in vitro techniques. Unlike the mitochondrial precursors which contain transient presequences for localization in mitochondria, the ADP/ATP carrier protein is imported into the organelle inner membrane without any apparent proteolytic processing (Pfanner and Neupert, 1987a). Early studies revealed that the binding of the soluble AAC protein prepared in a cell-free translation lysate was mediated by a protease-sensitive component which appeared to be distinct from that required for the efficient import of a presequence-containing mitochondrial precursor (Zwizinski et al., 1984). These studies and more recent analyses have clearly defined the mitochondrial apparatus required for binding and initial translocation of the AAC protein into mitochondria (Schwaiger et al., 1987). This receptor appears to be distinct from that mediating the binding of the F1-ATPase β-subunit precursor. These studies demonstrated that binding of the soluble AAC protein required a trypsin-sensitive component which was easily removed from mitochondria. Under conditions which have been documented to solubilize selectively the mitochondrial outer membrane (Schwaiger et al., 1987), the release of the ADP/ATP carrier precursor paralleled the release of the outer membrane protein porin.

Mitochondrial binding and insertion of the AAC protein into the mitochondrial inner membrane have been resolved into five distinct steps based on the combination of protease protection, alkalai extraction, temperature shift, and chromatographic studies (Pfanner and Neupert, 1987b). The soluble ADP/ATP carrier protein (stage 1) can bind to the mitochondrial outer membrane (stage 2) in the absence of a membrane potential to a protease-sensitive component. Following this binding, the AAC protein is sequestered in some manner (stage 3) such that it is no longer accessible to proteinases.
outside mitochondria. The stage 3 translocational intermediate is not fixed into a transmembrane complex in the bilayer because it can be solubilized by alkanl extraction. Entry or association of the translocational intermediate presumably into regions of contact between the outer and inner membrane (stage 4) is followed by assembly as the inner into the inner membrane (stage 5). This last step is characterized by an alkanl extraction-resistant form of the AAC protein. The presence of stage 4 and 5 forms of the ADP/ATP carrier in mitochondria is increased in the presence of a membrane potential across the mitochondrial inner membrane. This observation has led to the model that the membrane potential is required for the later assembly steps in the translocator import pathway.

At this time, there are no data on the sequences of the AAC protein which are involved in any step of this import sequence, and it is not known if different regions of the translocator participate in different steps. The AAC protein in yeast which is 308 amino acids in length is homologous to solute carrier proteins from other mitochondrial sources (Adrian et al., 1986). Earlier gene fusion studies have revealed that the first 115 residues of the AAC protein are sufficient to target α-galactosidase fused to it to mitochondria in vivo (Adrian et al., 1986). In order to examine directly the role of the first 115 residues in mitochondrial import, we have characterized the behavior of gene fusions between different amino-terminal lengths of the AAC protein in this region and a soluble reporter protein mouse dihydrofolate reductase. DHFR is a soluble cytoplasmic protein which does not bind to mitochondria unless it contains the appropriate mitochondrial import signals at its amino terminus (Hurt et al., 1984, 1985).

This study demonstrates that, unlike the basic hydrophilic mitochondrial import signals of presequences which are located at the extreme amino terminus, the import signal for the AAC protein is within a region which begins 72 residues from the amino terminus of the protein. In addition, this analysis indicates that the first 111 residues consisting of a putative membrane domain (residues 72–97) and a highly charged region (residues 97–111) are sufficient to deliver the protein only to an intermediate stage (stage 3) in the import pathway of the AAC protein. This stage 3 intermediate is found even in the presence of an energized membrane, conditions which promote transport of the complete AAC protein to the inner membrane. Thus, the import of the AAC protein to its final destination in the mitochondrial inner membrane may involve protein determinants distal to the first 111 residues of the protein.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Mitochondria were isolated from Saccharomyces cerevisiae strain D273-10B (MATa) grown in semisynthetic salts, 2% lactate medium (Du aum, 1982) to an optical density (600 nm) of 1.0–1.2. Yield was about 5 g of cells/liter. Escherichia coli MC1066 (F- lacX74 galU galK rpsL hsdR trpC9830 leuB60 pyrF74: Tn5) (Casadaban and Cohen, 1980) was used for amplification of plasmids. Ampicillin was added to media to select ampicillin-resistant transformants (Maniatis et al., 1982). E. coli JM101 (F- lac pro supE trd36) was used for M13 sequencing.

**DNA Methods and Construction of Plasmids**—The HindIII-Sall fragment of pFD11 (Simonsen and Levinson, 1989), containing 187 codons of mouse dihydrofolate reductase DNA, was inserted into the multilinker region of pTD2 (Genescribe). The pTD2 vector was opened at the unique Sall site of pUC19 (International Biotechnologies, Inc.). The pTD2 vector was opened at the unique HindIII site, and the HindIII fragment of pTD2: T Z151 was inserted (Fig. 1). The construct thus generated, pT2D, was linearized at the unique BamHI site located at the junction of the ADP/ATP carrier and lacZ DNA. Limited Bal31 digestion was followed by restriction at PstI, filling in with T, DNA polymerase and ligation with T, DNA ligase, generating the pT vector series. The HindIII fragments of the pT2 vectors were sized on 1.5% agarose gel. The 2 kDa plasmid was inserted at Sall and transcribed with T T polymerase (Chen and Douglas, 1987). Transcripts were translated in reticulocyte lysate (Promega Biotech) in the presence of [35S]methionine (Du Pont-New England Nuclear). Those pT2 vectors that translated a protein larger than DHFR were sequenced by the dideoxy method (Sanger et al., 1980). Translation was used in vitro to be used in in vivo import to be frozen in liquid nitrogen and stored at -70°C. DNA techniques were as described by manufacturers or according to Maniatis et al. (1982). ATP1 (Takeda et al., 1986) and full-length AAC1 (Adrian et al., 1986) were in pT2 transcription vectors. The vectors were linearized at a BamHI site in the ribulokinase, transcribed, and translated as described above.

**Mitochondrial Subfractionation**—Mitochondria were isolated (Daum et al., 1982) and suspended in 0.4 ml of 0.6 M mannitol, 10 mM Tris-Cl (pH 7.4). The import reaction consisted of 200 μl of mitochondria (1–2 mg of total protein) and 75 μl each of reticulocyte lysate containing 35S-labeled proteins. Final volume was 1.0 ml. The incubation of the import reaction (Gasser et al., 1982) included 0.6 M mannitol, 20 mM HEPES/KOH (pH 7.4), 1 mM ATP, 1 mM MgCl2, 5 mM phosphoenolpyruvate, 40 units of pyruvate kinase, 1 mM dithiothreitol. After incubation at 30°C for 30 min, the import mixture was treated with 250 μg/ml proteinase K (Sigma, Type XI) at 0°C for 30 min. PMSF was added to 1 mM final concentration. Mitochondria were then washed in a 1/20 dilution (0.6 ml of 20% sucrose) in 10 mM Tris-Cl (pH 7.4). The samples were centrifuged in a Beckman Microfuge at 13,000 g for 10 min. The pellet was resuspended in 80 μl of 0.6 M mannitol/HEPES (pH 7.4) to which 400 μl of cold 10 mM Tris-Cl (pH 8.0), 1 mM PMSF was added. Mitochondria were incubated for 10 min at 0°C and resolubilized by centrifugation at 13,000 × g for 10 min. The supernatant contained proteins of the intermembrane space. The pellet was resuspended in 0.6 M sucrose, 10 mM Tris (pH 7.4), 3 mM MgCl2, 3 mM ATP, 1 mM PMSF and sonicated twice for 60 s each with a 60-s interval using a Branson sonifier (setting 1, 0.1% duty). Membranes were separated from soluble proteins at 107,000 × g for 20 min at 4°C. Aliquots from each stage of the subfractionation procedure were suspended in 4 × SDS sample buffer and analyzed by gel electrophoresis using a 12.5% acrylamide-SDS gel (Laemmli, 1970). After electrophoresis, the gel was treated for fluorography using sodium salicylate (Chamberlain, 1979). The gel was dried down, and a fluorograph was made at -70°C using Kodak A-AF film.

Quantitation of the relative amounts of labeled protein present within an experiment was determined by scanning of fluorograms of gel following different times of exposure with a scanning densitometer (B-C Apparatus Corp.) and quantitating the relative areas under the peaks.

**Binding and Import of Labeled Proteins**—Mitochondria were isolated (Du aum et al., 1982) and resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH (pH 7.2) (Pfanner and Neupert, 1987b). The import reaction consisted of 3.5–7 μl of reticulocyte lysate and 20 μl (10–20 μg) of mitochondria/100 μl of total volume. The binding and import reactions contained, in addition, a buffer consisting of 250 mM sucrose, 10 mM MOPS/KOH (pH 7.2), 80 mM KCl, 5 mM MgCl2, and 3% bovine serum albumin (Pfanner and Neupert, 1987b). Inhibitors of mitochondrial protein, valinomycin and oligomycin (both from Sigma), were added from 100 × ethanol solutions where indicated. The import reactions were incubated at 25°C for 25 min and incubated into equal volumes. The reaction was added to 500 μl SUV1 and 30 μl of 35S-labeled protein, 25 μg/ml proteinase K for 30 min on ice. PMSF (1 mM) was then added to the proteinase K-digested reactions, and incubation on ice continued for an additional 5 min. Mitochondria were resolubilized through 0.6 ml of 20% sucrose, 10 mM Tris-Cl (pH 7.4) at 13,000 × g for 10 min. The pellet was resuspended in 4 × SDS sample buffer and analyzed as described above.

**Alkaline Extraction of Protease-treated Mitochondria**—In order to assess localization of hybrid proteins to soluble or membrane compartments, mitochondria were incubated with 35S-labeled proteins and treated with proteinase K as described above. Following incubations with PMSF, the mitochondria were washed once in 0.1 M Na2CO3 (pH 11.5) (Fujiki et al., 1982a, 1982b) at a concentration of 100–200 μg/ml, and incubated for 30 min on ice. Membranes were sedimented at 257,000 × g for 1 h. The supernatant was neutralized, and all samples were suspended in...
4 × SDS sample buffer for gel analysis. A fluorograph was made of the dried gel as described above.

RESULTS

Gene Fusions to AACl—The gene encoding the yeast ADP/ATP carrier protein was isolated by genetic complementation of the PET9 or OP1 mutation in earlier studies (O’Malley et al., 1982). Characterization of this gene has shown that it exhibits >70% identity with AAC proteins characterized from different organisms (Adrian et al., 1986). In this study, we have renamed this gene AACl to designate it by more conventional nomenclature as the gene encoding the ADP/ATP carrier protein. Expression of AACl-lacZ gene fusions in yeast revealed in earlier work that the first 115 residues of the ADP/ATP carrier were sufficient to direct the hybrid protein to mitochondria (Adrian et al., 1986).

In order to examine the role of these amino-terminal residues in further detail, selected gene fusions encoding various amino-terminal lengths of this region fused to mouse dihydrofolate reductase were constructed (Fig. 1) for analysis in an in vitro import assay (see “Experimental Procedures”). The recipient transcription vector containing the mouse DHFR gene was constructed by ligating a 1.2-kilobase HindIII-SalI fragment from plasmid pFD11 (Simonsen and Levinson, 1983) adjacent to the bacteriophage T7 promoter in plasmid pT7-2. This transcription plasmid places the HindIII site, which is 27 base pairs upstream of the DHFR translational start, adjacent to the T7 promoter. Transcription and translation of this vector yield a full-length DHFR translation product. This vector also serves as a recipient for a 0.5-kilobase HindIII fragment containing 115 codons of AAC fused to 641 codons of E. coli lacZ. This AAC-lacZ fragment was derived from plasmid TZ115 described previously (Adrian et al., 1986). The resulting plasmid, pTZD, contains 115 codons of AAC-lacZ-coding sequence proximal to the T7 promoter and separated from DHFR sequenced by 2 kilobases of lacZ DNA (see Fig. 1). Digestion of BamHI-restricted DNA with the processive exonuclease Bal31 was followed by Pst digestion and fill-in. This resulted in a family of gene fusions between AACl and DHFR which retained a HindIII site adjacent to the fusion joint (see “Experimental Procedures”).

Gene fusions between AACl and DHFR were screened for continuous reading frame using transcription/translation assays as well as DNA sequence analysis. In this study, three of many AACl-DHFR fusions characterized in this manner were isolated for further study. Fig. 2 depicts the fusions harboring 21, 72, and 111 codons of AAC fused to DHFR. In each case, a linker region encoding the sequence XHASLILEFAI is present in the fusion protein. The translation products of these gene fusions translated in reticulocyte lysate were 24, 29, and 32 kDa for the 21AAC-DHFR, 72AAC-DHFR, and 111AAC-DHFR forms of the hybrid proteins, respectively. Since each of these constructions retained the initiator AUG

![Fig. 1. Construction of pTD transcription vector series used for in vitro import studies.](image-url)

Details of the TZ115 construct containing the first 115 amino acids of the yeast ADP/ATP carrier and all of the E. coli lacZ gene except the first eight codons are published (Adrian et al., 1986). The vector containing full-length mouse DHFR, pFD11, is as described (Simonsen and Levinson, 1985). The pT7-based constructs all retained the T7 RNA polymerase promoter. The EcoRI-SstI fragment of TZ115 was inserted into pUC19, and the HindIII-SalI fragment of pFD11 was moved into the HindIII site of pT7-2. Subsequently, the HindIII fragment of pUC19:TZ115 was moved into the HindIII site of pT7-2:DHFR. Linearization of the pTZD vector thus generated at the BamHI site was followed by limited Bal31 digestion and release of lacZ DNA at the PstI site. Following T7 DNA polymerase fill-in of ends, the vector was ligated to form the pTD vector series, encoding various lengths of AACl upstream of DHFR. Constructs in which the start codon of AAC is in-frame with the start codon of DHFR were analyzed by transcription/translation, followed by DNA sequence analysis. kb, kilobase.
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of the dihydrofolate reductase, internal initiation yielded variable amounts of the complete DHFR protein (Fig. 3, lanes A, D, and G) in addition to the AAC-DHFR fusion product. Ligation of a HindIII-BglII fragment encoding the full-length ADP/ATP carrier protein directly into pT7-2 yielded the full-length AAC gene product (M, 34,000). For these studies, the ATP1 gene encoding the F1-ATPase \( \alpha \)-subunit precursor served as a control to establish distinctions between the import of a presequence-containing protein destined for the mitochondrial matrix and that for transmembrane insertion at the inner membrane (Takeda et al., 1986). The precursor of the F1-ATPase \( \alpha \)-subunit is a protein 61 kDa in size which is processed to a mature protein of 58 kDa by a metalloprotease in the mitochondrial matrix (Takeda et al., 1986).

Binding of the AAC Protein to Energized Mitochondria Requires Residues beyond the First 72 Amino Acids—AAC-DHFR gene fusion products containing different amino-terminal lengths of the AAC protein were incubated with isolated mitochondria in an \textit{in vitro} mitochondrial import reaction at 25 °C for 30 min (see “Experimental Procedures”). Following incubation, mitochondria were separated from the reticulocyte lysate reaction mixture by sedimentation through a sucrose cushion. Shown in Fig. 3 is an autoradiogram of mitochondrial pellet fractions for each construct following SDS gel separation. The 111AAC-DHFR protein exhibited both binding and import to the same extent observed for the AAC protein alone (see below). However, under these conditions, less than 2% binding of the input protein could be observed for either the 21AAC-DHFR or 72AAC-DHFR protein (see “Experimental Procedures”). In each case, the bound 21AAC-DHFR and 72AAC-DHFR proteins remained completely accessible to added proteinase K. DHFR alone under these conditions exhibited no detectable binding to mitochondria. In separate experiments, even under conditions in which the shorter forms of the hybrid protein were incubated in large excess over those used in Fig. 3, no significant binding could be observed.

The ATP1 gene product under these conditions exhibited import to a protease-protected location and processing by the matrix metalloprotease (see below) as previously described (Takeda et al., 1986). The complete lack of binding observed for the shorter forms of the AAC-DHFR proteins indicated that the binding observed for the 111AAC-DHFR protein represented a specific association with components on the mitochondrial membrane. Earlier studies indicated that proteinaceous components on the surface mediate binding of the AAC protein (Zwizinski et al., 1984; Schwaiger et al., 1987).

Binding and Import of 111AAC-DHFR in Mitochondria—To define further the interaction of 111AAC-DHFR with the mitochondrial surface and its relationship to the AAC import pathway, we took advantage of the observation that binding of the ADP/ATP carrier protein to mitochondria at 2 °C prevented its movement from stage 2 to stage 3. The bound AAC protein is not moved from the mitochondrial surface to some internal location and therefore remains accessible to protease (Pfanner and Neupert, 1987b). As shown in Fig. 4, when the 111AAC-DHFR protein is bound to mitochondria at 2 °C, it remains accessible to subsequently added proteinase K. Essentially, the same protease accessibility is observed for the bound AAC protein under these conditions (Fig. 4, lane C). As a control, the ATP1 gene product which was also included in the incubation with AAC exhibited a translocation intermediate form of the mature F1-ATPase \( \alpha \)-subunit at 2 °C. In this case, the mature protein spanning both the inner and outer membranes remained accessible to proteinase K (Fig. 4, lane C). The incubations performed here were not optimal for ATP1 import since the only ATP present in the import incubation was that added with the translation lysate. However, at 2 °C, only 7% of the input ATP gene product was bound to mitochondria. Upon a shift to 25 °C, there was increased F1-ATPase \( \alpha \)-subunit precursor binding; however, in the presence of valinomycin to block maintenance of a membrane potential prior to the shift from 2 to 25 °C, very little bound \( \alpha \)-subunit exhibited protease protection (Fig. 4, lane E). However, at this temperature and in the absence of a membrane potential, both the 111AAC-DHFR and AAC proteins exhibited efficient internalization to a protease-protected space (Fig. 4, lane E).

111AAC-DHFR Is Blocked at an Intermediate Stage of Import—it was noteworthy that essentially all the bound AAC and 111AAC-DHFR proteins were efficiently internalized even under conditions in which the sole source of ATP for energizing mitochondria was relatively low (~5 μM ATP). In a previous study (Pfanner and Neupert, 1987b), it was shown that efficient localization of the AAC protein to a space
Fig. 4. Protease accessibility of ATP1, full-length AAC, and 111AAC-DHFR proteins following binding at 2 °C and shift to 25 °C. Mitochondria were incubated in reticulocyte lysate in a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, and 10 mM MOPS/KOH (pH 7.2) for 30 min on ice. One aliquot was removed, 1 mM PMSF was added, and mitochondria were reisolated (lanes B). To a second aliquot, 25 μg/ml protease K was added, incubation on ice was continued 30 min, 1 mM PMSF was added, and mitochondria were reisolated (lanes C). To a third aliquot, 1 μM valinomycin was added, and the mixture was incubated at 25 °C for 30 min. After cooling on ice for 5 min, the reaction was divided, 1 mM PMSF was added to half, and mitochondria were reisolated (lanes D). To the other half, 25 μg/ml protease K was added, incubation on ice was continued for 30 min, 1 μM PMSF was added, and mitochondria were reisolated (lanes E). Lanes A represent 20% of reticulocyte lysate used for incubation with mitochondria; p, F₁,ATPase precursor form; m, F₁,ATPase mature form.

protected from protease (stage 3) occurred in the absence of a membrane potential. However, assembly of the protein into a transmembrane-bound form could occur only under conditions in which the mitochondrial inner membrane was energized. In this study, the ability to distinguish AAC protein in a transmembrane-bound form versus a more peripheral association with the membrane in a protease-protected space took advantage of an alkaline extraction protocol for resolving peripheral and integral membrane proteins (Pfanner et al., 1987b). It has been shown that treatment of membranes with 100 mM sodium carbonate will release soluble and peripheral membrane proteins from the bilayer, but not integral membrane proteins which remain pelletable with the membrane fraction (Fujiki et al., 1982a, 1982b). In this study, we used the F₁,ATPase α-subunit as a marker for a peripheral membrane protein. When this subunit is either assembled with other subunits into the F₁,ATPase or associated with membrane in the unassembled state, it can be released in soluble form from the organelle membrane by sonication or salt treatment (Todd and Douglas, 1981).

As shown in Fig. 5, mitochondria which were either untreated or de-energized with 20 μM oligomycin plus 1 μM valinomycin were mixed with reticulocyte lysate containing either the F₁,ATPase α-subunit precursor and AAC protein or the 111AAC-DHFR protein. Following the import reaction, mitochondria were treated with protease K and then pelleted through a sucrose cushion. In each case, half of the mitochondrial pellet was taken directly for gel analysis (lanes B and E), and the other half was extracted with 100 mM sodium carbonate and then resolved by centrifugation into a pellet (lanes D and G) and supernatant (lanes C and F). In de-energized mitochondria (lanes B–D), the F₁,ATPase α-subunit is not observed with the protease-treated mitochondria. This is anticipated since the membrane potential is required to drive import of a presequence-containing precursor such as the F₁,ATPase α-subunit precursor into a protease-protected location in the organelle (see also Fig. 4). Furthermore, binding of presequence-containing mitochondrial precursors to mitochondria in the absence of an energized inner membrane is very low or, in some cases, does not occur at all (Pfanner et al., 1987b). On the other hand, both the AAC and 111AAC-DHFR proteins exhibited efficient import into a protease-protected space of the de-energized organelle (lane B). In fact, the level of import of the two AAC proteins was essentially the same as that observed in the absence of inhibitors. Under these latter conditions of an energized membrane, efficient import and protection of the F₁,ATPase α-subunit were readily apparent (lane E).

When the protected forms of the proteins were characterized for their solubility upon alkali extraction, we observed that approximately 50% of the AAC protein was extractable in de-energized mitochondria (Fig. 5, lane C) or in energized mitochondria (lane F). Full-length AAC proteins remained entirely with the mitochondrial membrane pellet from energized mitochondria (lane G). These extraction conditions quantitatively solubilized the peripherally associated F₁,ATPase α-subunit (lane F). The extractability of the AAC protein with sodium carbonate following import into de-energized mitochondria was qualitatively identical to that reported earlier (Pfanner and Neupert, 1987b). These observations led to the proposal that an energized membrane was required to drive the AAC protein to its alkali-nonextractable transmembrane-associated form. It has been further proposed that the alkali-extractable translocational intermediate form of the AAC protein represented its association with a proteinaceous component either on or within the mitochondrial
membranes (Pfanner et al., 1988). The protease-protected 111AAC-DHFR protein, however, was readily extractable to the same extent by sodium carbonate from either energized or de-energized mitochondria (compare lanes C and F). Thus, the presence of an energized inner membrane which dramatically influenced import of the F1-ATPase α-subunit precursor and transmembrane insertion of the AAC protein into the membrane had no discernible effect on the localization of the 111AAC-DHFR protein.

111AAC-DHFR Translocational Intermediate Is Membrane-bound—A mitochondrial subfractionation experiment was performed to determine if the 111AAC-DHFR protein was either localized as a soluble translocation intermediate within one of the two soluble compartments of mitochondria or was associated with the membrane. Following incubation of the ATP1, AAC1, and 111AAC-DHFR gene products with mitochondria, the incubation mixture was treated with proteinase K and separated into mitochondrial and supernatant fractions. Subfractionation of the proteinase K-treated mitochondria into the intermembrane space, membrane, and matrix fractions followed (Fig. 6). Throughout the subfractionation, the 111AAC-DHFR protein cofractionated with the membrane fraction in the same manner as the full-length AAC1 protein (lanes D, F, and H) even under conditions which utilized relatively high ionic strength. In addition, this cosedimentation with membranes occurred even under conditions of relatively harsh hypotonic treatment to release components of the intermembrane space. In this experiment, the hypotonic lysis conditions also released soluble mature F1-ATPase α-subunit (lane E), which has previously been shown to reside in the mitochondrial matrix. Although these observations do not define which of the two membranes the 111AAC-DHFR protein is localized to, they do show that this internalized intermediate remains firmly associated with the membrane fraction in a protected space.

DISCUSSION

Recent analysis of the protein signals which direct protein localization into mitochondria has focused on the transient sequences which specify delivery to the matrix or the intermembrane space (for review, see Douglas et al., 1986). The matrix delivery signals are hydrophilic sequences capable of generating structures with a high hydrophobic movement (Von Heijne, 1986; Roise et al., 1986). These protein elements at the amino terminus of a protein have been shown to be necessary and sufficient to catalyze the entry of any protein fused carboxyl-terminal to it through both membranes of the mitochondria. The most recent analysis of the matrix delivery pathway suggests that this transmembrane delivery occurs through a hydrophilic membrane environment (Pfanner et al., 1987b). Addition of an appropriate second localization signal, usually a transmembrane-spanning sequence, adjacent to the matrix targeting signal serves to localize further the protein either to the outer membrane (Hase et al., 1984) or to the intermembrane space (Van Loon et al., 1986; Van Loon and Schatz, 1987; Hartl et al., 1986). These hydrophilic secondary localization signals act to direct export of an intermediate form of the protein from the mitochondrial matrix (Hartl et al., 1986).

With the exception of the special case of cytochrome c, the protein elements characterized to date which direct protein localization to sites within mitochondria beyond the outer membrane are transient and are usually dispensed with once they have catalyzed their localization function. Thus, removal of targeting and localization elements at the amino-terminal end of the protein has been proposed to provide a vectorial basis for their biogenesis in the bilayer. In its simplest form, the localization of proteins utilizes different determinants at the amino-terminal end of the protein which are dispensed with following their utilization.

The ADP/ATP carrier protein of the mitochondrial inner membrane is representative of a class of integral membrane proteins which are localized within mitochondria. First, there are no apparent post-translational processing steps which participate in its localization pathway. Second, the extreme amino-terminal end of the ADP/ATP carrier does not exhibit an amino acid sequence with the potential for forming structures which have been documented previously to mediate protein import (Hurt et al., 1984; Allison and Schatz, 1986; Vassarotti et al., 1987). Earlier work from this laboratory has shown that the first 115 residues of the AAC protein are sufficient to deliver a hybrid gene product into mitochondria.
protein is localized as an intermediate in the same manner as the 111AAC-DHFR protein in this study. Therefore, we propose that the inability of the hybrid protein to insert into the bilayer beyond stage 3 was most likely due to the lack of additional sequences more carboxy-terminal to first 111 residues, perhaps a second transmembrane-spanning sequence. It is noteworthy in this regard that the insertion of M13 procoat protein across the bilayer into a transmembrane form requires two hydrophobic membrane-spanning sequences working together. Analysis of the insertion of the M13 procoat into the E. coli inner membrane has shown that the introduction of mutations (Kuhn et al., 1986a) or deletions (Kuhn et al., 1986b) to remove or break up the second transmembrane-spanning domain severely delays the kinetics of or block entirely the membrane localization of the protein. We suspect that the same principles documented for insertion of the M13 procoat protein into the bilayer may define the movement of the AAC protein into the bilayer beyond stage 3. Studies to define both the role of additional transmembrane-spanning domains in stable membrane insertion and the component to which the stage 3 intermediate is complexed are currently in progress.

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