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Distinct Steps in the Import of ADP/ATP Carrier into Mitochondria*

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Transport of the precursor to the ADP/ATP carrier from the cytosol into the mitochondrial inner membrane was resolved into several consecutive steps. The precursor protein was trapped at distinct stages of the import pathway and subsequently chased to the mature form. In a first reaction, the precursor interacts with a protease-sensitive component on the mitochondrial surface. It then reaches intermediate sites in the outer membrane which are saturable and where it is protected against proteases. This translocation intermediate can be extracted at alkaline pH. We suggest that it is anchored to the membrane by a so far unknown proteinaceous component. The membrane potential $\Delta\psi$-dependent entrance of the ADP/ATP carrier into the inner membrane takes place at contact sites between outer and inner membranes. Completion of translocation into the inner membrane can occur in the absence of $\Delta\psi$. A cytosolic component which is present in reticulocyte lysate and which interacts with isolated mitochondria is required for the specific binding of the precursor to mitochondria.

The bulk of mitochondrial proteins are synthesized on cytoplasmic polysomes as water-soluble precursor proteins (for review, see Hay *et al.*, 1984; Harmey and Neupert, 1985; Pfanner and Neupert, 1987). Many precursor proteins are transported in the form of high molecular weight aggregates in the cytosol (Zimmermann and Neupert, 1980; Schmidt *et al.*, 1983). The import of precursors into mitochondria requires proteinaceous components on the mitochondrial surface which may act as receptor sites (Hennig and Neupert, 1981; Gasser *et al.*, 1982; Hennig *et al.*, 1983; Zwizinski *et al.*, 1983, 1984; Riezman *et al.*, 1983; Pfaller *et al.*, 1985; Schmidt *et al.*, 1985; Hartl *et al.*, 1986). Transport into or across the inner membrane needs energization of the membrane (Schleyer *et al.*, 1982; Gasser *et al.*, 1982; Kolanski *et al.*, 1982). The required energy form is the electrical component (membrane potential $\Delta\psi$) of the total protonmotive force (Pfanner and Neupert, 1985). In addition, nucleoside triphosphates are required for import of mitochondrial precursor proteins (Pfanner and Neupert, 1986).[1] The role of the membrane potential could be an electrophoretic effect on positively charged domains in the precursor proteins (Pfanner and Neupert, 1985; Roise *et al.*, 1986; von Heijne, 1986). For several precursor proteins which are synthesized with amino-terminal peptide extensions import via translocation contact sites between outer and inner membranes has been demonstrated (Schleyer and Neupert, 1985; Hartl *et al.*, 1986).

Many precursors carry amino-terminal presequences which are cleaved off during or after translocation by the metal-dependent processing peptidase in the mitochondrial matrix (Böhni *et al.*, 1980, 1983; Conboy *et al.*, 1982; McAda and Douglas, 1982; Miura *et al.*, 1982; Zwizinski and Neupert, 1983; Schmidt *et al.*, 1984). The presequences apparently contain sufficient information for the targeting of proteins to mitochondria (Hurt *et al.*, 1984a, 1984b, 1985; Horwich *et al.*, 1985; Emr *et al.*, 1986; Keng *et al.*, 1986; van Loon and Young, 1986a). The presequences may also be important for the intramitochondrial sorting (van Loon *et al.*, 1986b; Hartl *et al.*, 1986).

The functional form of the ADP/ATP carrier, an integral protein of the inner mitochondrial membrane, is a dimer of two identical subunits (M_r 32,000) (Klingenberg *et al.*, 1978). The precursor to the ADP/ATP carrier is synthesized without an amino-terminal peptide extension (Zimmermann *et al.*, 1979; Arends and Sebald, 1984). It can be bound to de-energized mitochondria and then imported from the bound state after re-energization of the mitochondria (Zwizinski *et al.*, 1983; Pfanner and Neupert, 1985). The first 115 amino acid residues (about one-third of the protein) appear to carry sufficient information for the targeting to mitochondria (Adrian *et al.*, 1986). Unlike the precursor, the assembled ADP/ATP carrier in the inner membrane is able to bind the specific inhibitor carboxyatractyloside and to pass over hydroxylapatite (Schleyer and Neupert, 1984). This serves as a convenient assay for import and assembly of the ADP/ATP carrier.

We studied the interaction of *in vitro* synthesized precursor with de-energized and energized mitochondria at different temperatures. Several consecutive steps of the translocation of the precursor across the mitochondrial membranes were defined. These include: interaction with a protease-sensitive component on the mitochondrial surface; a translocational intermediate in the outer membrane; and import via contact sites between outer and inner membranes. In order to define the various steps, we have named the distinct forms of the ADP/ATP carrier from stage 1 to stage 5, according to the numbers used in Fig. 9. Furthermore, the role of putative cytosolic cofactors for the import of the ADP/ATP carrier was investigated.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria—Neurospora crassa (wild type 74A) was grown as described previously (Schleyer *et al.*, 1982). Mitochondria were isolated either by differential centrifugation (Pfanner and Neupert, 1985) or by Percoll density-gradient centrifugation (Hartl *et al.*, 1986) as described except that the hyphae were ground for 30–60 s. After washing in SEM (250 mM sucrose, 1 mM EDTA, 10 mM

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[1] N. Pfanner and W. Neupert, unpublished data.

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MOPS,[2] adjusted to pH 7.2 with KOH), mitochondria were suspended in SEM at a protein concentration of 4 mg/ml. Protein determination was done according to Bradford (1976).

Synthesis of Precursor Proteins in Rabbit Reticulocyte Lysates—Mitochondrial precursor proteins were synthesized *in vitro* in rabbit reticulocytes lysates (Pelham and Jackson, 1976) in the presence of [^{35}S]methionine (specific radioactivity, 500 Ci/mmol, Amersham Buchler) and *Neurospora* poly(A^+) RNA as described (Schleyer *et al.*, 1982). To produce "unlabeled lysates" containing precursor proteins which were not radiolabeled, [^{35}S]methionine was omitted. "No synthesis lysates" lacking mitochondrial precursor proteins were prepared as described (Pfanner and Neupert, 1985). Preparation of postribosomal supernatants and supplementation was performed as published (Schleyer *et al.*, 1982). To obtain "G-25 lysates," columns containing Sephadex G-25M in a bed volume of 9 ml (Pharmacia P-L Biochemicals) were equilibrated with a buffer containing 250 mM sucrose, 15 mM MOPS, 80 mM KCl, and 5 mM $MgCl_2$, adjusted to pH 7.2 with KOH; then 1 ml of postribosomal supernatant was supplemented with unlabeled methionine and passed over the column. The red pass-through was collected.

Binding and Import of ADP/ATP Carrier—Mitochondria and reticulocyte lysate containing precursor proteins were mixed and incubated. Where indicated, a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, and 3% (w/v) BSA, adjusted to pH 7.2 with KOH, was included. Antimycin A (Sigma) and oligomycin (Sigma) (8 and 20 μM final concentrations, respectively) were added from a 100-fold concentrated stock solution in ethanol or, when further solutions containing ethanol were included in the reaction mixture, from a 200-fold concentrated stock solution. Valinomycin (Sigma) (0.5 μM final concentration) was added from a 200-fold concentrated stock solution in ethanol. Potassium ascorbate (Merck) (pH 7) and TMPD (Sigma) (8 and 0.2 mM final concentrations, respectively) were added from 50-fold concentrated solutions in water. The samples were made chemically identical by adding the same amount of reagent-free solvent to the control samples. In several experiments, a cytosolic proteinase-inhibitor fraction of *N. crassa* (Schmidt *et al.*, 1984) was included (400 μg of protein of the proteinase-inhibitor fraction/mg of mitochondrial protein). Times and temperatures of incubations are given in the figure legends. Where indicated, mitochondria were reisolated by centrifugation for 12 min at 27,000 × *g*. Prior to the reisolation (preceding the immunoprecipitation), PMSF was added to a final concentration of 1 mM.

Protease Treatment—Samples were cooled to 0 °C, and then trypsin (EC 3.4.21.4; 10,000–13,000 BAEE (N^{α}-benzoyl-L-arginine ethyl ester) A_{253} units/mg of protein; treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Sigma T 8642) or proteinase K (EC 3.4.21.14; 20 units/mg; Boehringer Mannheim) was added from solutions in SEM as indicated in the figure legends. After incubation for 25 min at 0 °C, PMSF was added to a final concentration of 1 mM and, in the case of treatment with trypsin, a 20-fold weight excess (of the highest amount of trypsin used in the experiment) of soybean trypsin inhibitor (Sigma T 9003; chromatographically prepared) was included. Samples were further incubated for 10 min at 0 °C. When treatment with proteinase K followed treatment with trypsin, then PMSF was not added after the first incubation.

Assessment of Import of ADP/ATP Carrier by Hydroxylapatite Chromatography—Five minutes before the end of the 25 °C import incubation, carboxyatractyloside (Boehringer Mannheim) was added to a final concentration of 14 μM. After reisolation of mitochondria, lysis of mitochondria, and passage of the samples over hydroxylapatite as previously described (Schleyer and Neupert, 1984), ADP/ATP carrier was immunoprecipitated from the unbound fraction.

Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis—Immunoprecipitation of ADP/ATP carrier, SDS-polyacrylamide gel electrophoresis, fluorography (Chamberlain, 1979), and quantitation of immunoprecipitated ADP/ATP carrier were performed as described (Schleyer *et al.*, 1982; Pfanner and Neupert, 1985).

RESULTS

A Translocational Intermediate before the Entrance into the Inner Membrane—Precursor to the ADP/ATP carrier was synthesized in a rabbit reticulocyte lysate. The lysate was diluted with a buffer containing 3% BSA (Pfanner and Neupert, 1985), and isolated mitochondria were added that had been de-energized by addition of antimycin A and oligomycin (which inhibit at the level of complex III of the electron transport chain and the F_OF_1-ATPase, respectively; Nicholls, 1982; Wikstrom and Krab, 1982; Schleyer *et al.*, 1982). After 25 min at 25 °C, samples were cooled to 0 °C and treated with proteinase K (Fig. 1*A*) or trypsin (Fig. 1*B*). The bound precursor was largely resistant (70–80%) to proteinase K concentrations up to 40 μg/ml and to all trypsin concentrations tested (up to 270 μg/ml). At proteinase K concentrations higher than 40 μg/ml, the bound precursor was degraded whereas the protein imported into energized mitochondria was still protease resistant (Fig. 1*A*).

This treatment with high concentrations of proteinase K corresponds to the conditions which have been used before to test for import of ADP/ATP carrier into the inner membrane (Schleyer *et al.*, 1982; Zwizinski *et al.*, 1983; Pfanner and Neupert, 1985). Resistance to this treatment with proteinase K correlates well with a specific criterium for assembly of the ADP/ATP carrier, namely the carboxyatractyloside-dependent passage over hydroxylapatite (Schleyer and Neupert, 1984).

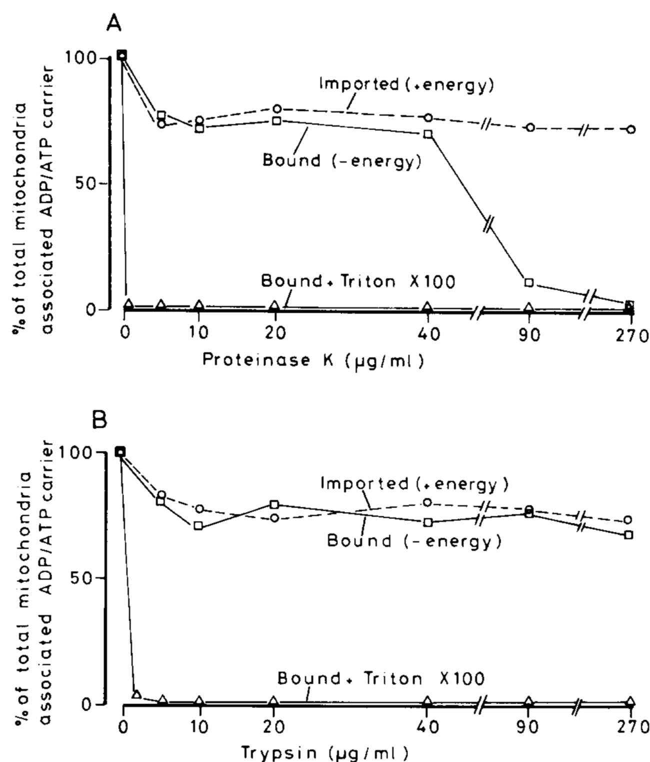

FIG. 1. **Protease sensitivity of various forms of ADP/ATP carrier.** Forty μl of reticulocyte lysate containing radiolabeled precursor proteins and isolated mitochondria (corresponding to 20 μg of mitochondrial protein) were incubated in the presence of either antimycin A and oligomycin (*Bound*) or ascorbate plus TMPD (*Imported*). The reaction mixture contained a buffer with 3% (w/v) BSA (see "Experimental Procedures") in a final volume of 400 μl. Samples were incubated for 25 min at 25 °C, cooled to 0 °C, and treated with proteinase K (*A*) or trypsin (*B*) at the indicated concentrations. From the samples (*Bound + Triton X100*), the mitochondria were reisolated, resuspended in BSA buffer containing 1% (w/v) Triton X-100, and kept at 0 °C for 15 min prior to the protease treatment; the ADP/ATP carrier was then immunoprecipitated. From the other samples, the mitochondria were reisolated after the protease treatment, and then ADP/ATP carrier was immunoprecipitated. The samples were resolved by SDS-polyacrylamide gel electrophoresis, and quantitation of the fluorograms was performed by densitometry.

[2] The abbreviations used are: MOPS, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.

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When the mitochondria were lysed with Triton X-100 prior to the protease treatment, the ADP/ATP carrier was completely digested even at very low protease concentrations (Fig. 1). Similarly, a high sensitivity to protease treatment was observed for the precursor in the reticulocyte lysate (not shown).

About 25% of the precursor associated with mitochondria was very sensitive to protease treatment. As shown below, this fraction represents unspecific (*i.e.* import-incompetent) association with mitochondria.

In order to determine whether the protease-resistant precursor associated with de-energized mitochondria (stage 3) is on the correct import pathway, the following experiments were performed. Precursor was bound to de-energized mitochondria at 25 °C. Mitochondria were reisolated and resuspended in BSA-containing buffer and then treated with trypsin (Fig. 2*A*) or proteinase K (Fig. 2*B*). By addition of ascorbate plus TMPD, which supplies electrons at the level of

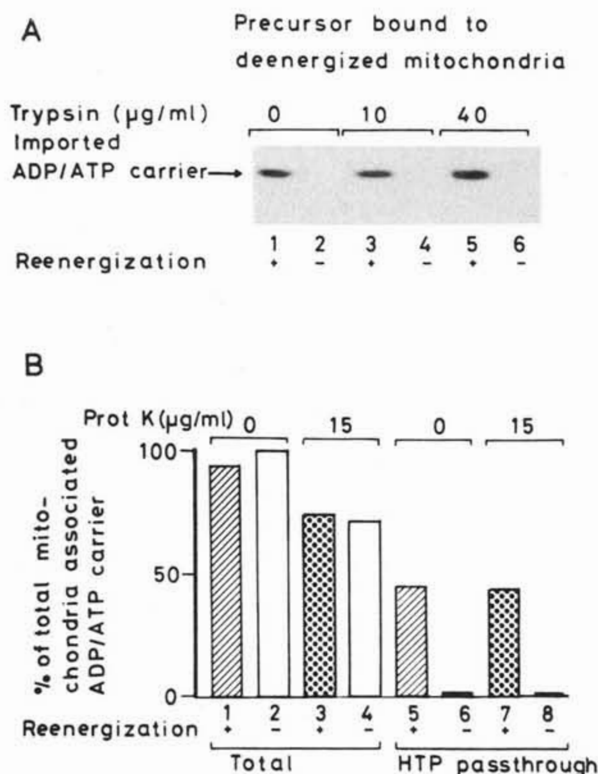

FIG. 2. **The protease-protected precursor bound to de-energized mitochondria is on the correct assembly pathway.** *A*, reticulocyte lysate (240 μl) and mitochondria (120 μg of mitochondrial protein) were incubated in the presence of antimycin A and oligomycin. After incubation for 25 min at 25 °C mitochondria were reisolated from the reaction mixture, resuspended in BSA-containing buffer in the presence of antimycin A and oligomycin, and divided into 6 aliquots of 300 μl. Samples 3–6 were treated with trypsin at the indicated concentrations; samples 1 and 2 received the same volume of SEM. Ascorbate and TMPD were added to samples 1, 3, and 5 (samples 2, 4, and 6 received water). After incubation for 15 min at 25 °C, mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography (see "Experimental Procedures"). Experiment B was performed as described for *A* with the following modifications. Reticulocyte lysate (320 μl) and mitochondria (160 μg of mitochondrial protein) were incubated. After reisolation, mitochondria were divided into 4 aliquots. Samples were treated with proteinase K (*Prot K*) (instead of trypsin) at the indicated concentrations. After the second reisolation, samples were halved. From one half total ADP/ATP carrier was immunoprecipitated (*columns 1–4*); from the other half assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography (*columns 5–8*).

complex IV of the electron transport chain, a membrane potential was established (Nicholls, 1982; Wikstrom and Krab, 1982; Schleyer *et al.*, 1982). The mitochondria were isolated again and tested for imported functional ADP/ATP carrier (stage 5). As a specific criterium for the assembly of ADP/ATP carrier the carboxyatractyloside-dependent passage over hydroxylapatite was used. The same amount of precursor was imported in the samples treated with protease (Fig. 2*A*, *lanes 3* and *5*; Fig. 2*B*, *column 7*) and in the untreated samples (Fig. 2*A*, *lane 1*; Fig. 2*B*, *column 5*). Taking into account that 55–60% of the assembled ADP/ATP carrier applied to a hydroxylapatite column is recovered in the pass-through under the conditions used (Schleyer and Neupert, 1984), practically all of the protease-resistant precursor could be imported into the inner membrane (Fig. 2*B*, *columns 3* and *7*). Similarly, when treatment with proteinase K at high concentrations or at temperatures above 0 °C (4–8 °C) was used as a criterium for determining the amount of imported ADP/ATP carrier, 70–80% of the bound precursor were found to be imported after re-energization of mitochondria (Pfanner and Neupert, 1985). On the other hand, none of the bound precursor was imported when the mitochondria were not re-energized (Fig. 2*A*, *lanes 2*, *4*, and *6*; Fig. 2*B*, *columns 6* and *8*). These results demonstrate that the protease-resistant precursor associated with de-energized mitochondria is on the correct import pathway and is in a location preceding the membrane potential-requiring step. Furthermore, all ADP/ATP carrier which was imported from the bound state after re-energization of mitochondria had originated in the protease-resistant form.

The Stage 3 Translocational Intermediate Is beyond the Import Step Which Requires a Protease-sensitive Component on the Mitochondrial Surface—Import of free precursor can be inhibited by pretreatment of mitochondria with low concentrations of protease (Zwizinski *et al.*, 1984). The question arises as to whether the protease-resistant bound precursor is past the stage of interaction of precursor with the protease-sensitive component on the mitochondrial surface.

De-energized mitochondria were incubated at 25 °C with reticulocyte lysate which did not contain *Neurospora* precursor proteins ("no synthesis lysate") (Fig. 3, *lanes 1–3*) or reticulocyte lysate containing unlabeled precursor proteins ("unlabeled lysate") (Fig. 3, *lanes 4–6*). The mitochondria were reisolated, resuspended in BSA-containing buffer, and treated with trypsin at the indicated concentrations (*lanes 2*, *3*, *5*, and *6*) or with trypsin which had been preincubated with trypsin inhibitor (*lanes 1* and *4*). The mitochondria were then re-energized at 25 °C to allow import of bound precursors. Finally, reticulocyte lysate containing ^{35}S-labeled precursor proteins ("^{35}S-lysate") was added, and a third incubation at 25 °C was performed. After reisolation of the mitochondria, the imported radiolabeled ADP/ATP carrier was determined. *Lanes 2* and *3* of Fig. 3 show that protease treatment of mitochondria under conditions where all of the specifically bound precursor (stage 3) remains unaffected (Fig. 2*A*, *lanes 3* and *5*, and Fig. 2*B*, *column 7*) strongly inhibits the import of free precursor (stage 1). This suggests that the protease-resistant bound precursor (stage 3) is beyond the interaction of precursor with the putative receptor on the mitochondrial surface.

The import of free precursor was also inhibited when unlabeled precursor was bound to mitochondria before protease treatment (Fig. 3, *lanes 5* and *6*). This excludes that the normally protease-sensitive component ("receptor") might be protected by bound precursor molecules against protease digestion.

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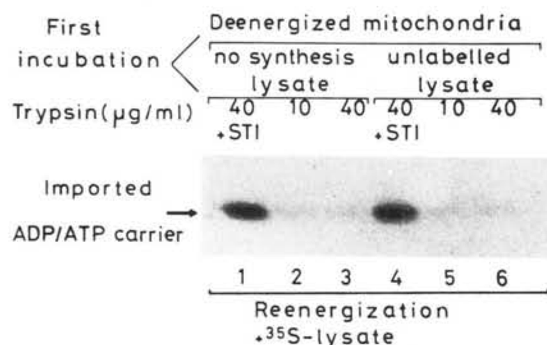

FIG. 3. **Pretreatment of mitochondria with protease inhibits the import of free precursor.** *no synthesis lysate* (150 μl) (*lanes 1–3*) or *unlabeled lysate* (150 μl) (*lanes 4–6*) was incubated with mitochondria (20 μg of mitochondrial protein) in the presence of antimycin A and oligomycin for 25 min at 25 °C (see "Experimental Procedures"). Mitochondria were reisolated and resuspended in 300 μl of BSA-containing buffer in the presence of antimycin A and oligomycin. Samples were treated with trypsin at the indicated concentrations (*lanes 2, 3, 5,* and *6*) or with trypsin which had been preincubated with a 20-fold excess (w/w) of soybean trypsin inhibitor (*STI*) for 10 min at 0 °C prior to the addition (*lanes 1* and *4*). Then ascorbate plus TMPD was added to all samples. After 5 min at 25 °C, 80 μl of reticulocyte lysate containing radiolabeled precursor proteins (*^{35}S-lysate*) were added, and the samples were further incubated for 25 min at 25 °C. Mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography.

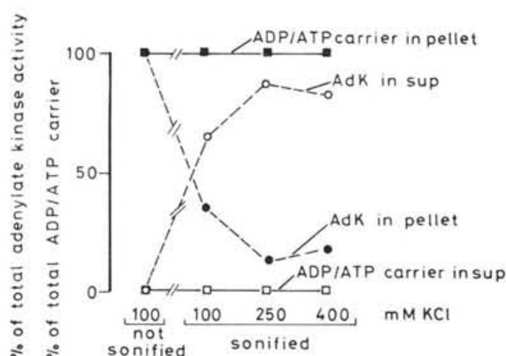

FIG. 4. **The protease-protected bound precursor is membrane associated.** Forty μl of reticulocyte lysate containing radiolabeled precursor proteins were incubated with mitochondria (50 μg of mitochondrial protein) in the presence of antimycin A and oligomycin for 25 min at 25 °C. Mitochondria were reisolated and suspended in BSA-containing buffer in the presence of antimycin A and oligomycin. The samples were treated with trypsin (10 μg/ml final concentration). Mitochondria were isolated again and suspended in 500 μl of SEM containing antimycin A, oligomycin, PMSF (0.5 mM final concentration), and KCl at the indicated concentrations. Samples were sonified at 0–4 °C with a Branson Sonifier with tapered microtip (setting 2.5, 30% duty, 2 × 60 s with an interval of 60 s); control samples (*not sonified*) were left on ice. PMSF was added to a final concentration of 1 mM. Samples were separated into pellets and supernatants (*sup*) by centrifugation for 90 min at 166,000 × *g*. Supernatants were brought to 1% (w/v) Triton X-100 and 5 mM EDTA, and ADP/ATP carrier was immunoprecipitated from both pellets and supernatants. In parallel samples, the enzyme activity of adenylate kinase (*AdK*) in pellets and supernatants was determined as described (Schmidt *et al.*, 1984).

In summary, the results demonstrate a translocational intermediate (stage 3) on the import pathway of the ADP/ATP carrier. The intermediate is beyond the initial interaction of the precursor with a proteinaceous receptor site on the mitochondrial surface but exists before the membrane potential-dependent entrance of the precursor into the inner membrane.

The Stage 3 Translocational Intermediate Is Membrane-associated but Can Be Extracted at Alkaline pH—Mitochondria were sonicated under conditions where soluble marker proteins were released into the supernatant (Schmidt *et al.*, 1984; Hartl *et al.*, 1986), as illustrated by the release of the intermembrane space marker adenylate kinase (Fig. 4). The protease-resistant translocational intermediate (stage 3), however, remained completely associated with the membrane fraction at all KCl concentrations applied (Fig. 4).

Incubation of membranes at alkaline pH (carbonate treatment) is known to release soluble proteins and peripheral membrane proteins into the supernatant and to leave integral proteins in the membrane fraction (Fujiki *et al.*, 1982a, 1982b). About 75% of the precursors bound to de-energized mitochondria could be extracted at pH 11.5, while 25% remained in the membrane fraction (Fig. 5, *column 1*). After binding of precursor, mitochondria were treated with protease under conditions which do not digest the stage 3 translocational intermediate. Under these circumstances, practically all of the precursor could be extracted by the carbonate treatment (Fig. 5, *column 2*). Mitochondria which had been pretreated with proteases were substantially reduced in the ability to import free precursor (see Fig. 3; Zwizinski *et al.*, 1984), although there was still binding of precursor to these mitochondria (about 25% of control). This binding apparently produced an import-incompetent species (*i.e.* unspecifically bound precursor) that was resistant to carbonate extraction (Fig. 5, *column 3*). Controls (Fig. 5, *columns 4* and *5*) showed that the imported and assembled ADP/ATP carrier (stage 5) was resistant to carbonate extraction as was expected for an integral membrane protein.

In summary, 70–80% of the precursor bound to de-energized mitochondria at 25 °C can be imported after re-energization of the mitochondria. This specifically bound precursor (stage 3) is membrane associated but can be extracted at alkaline pH. Thus, this translocational intermediate shows characteristics of a membrane protein not firmly integrated into the lipid phase. Scatchard analysis of the binding of the precursor at 25 °C indicated 1.7 pmol of specific binding sites/mg of mitochondrial protein (Schmidt *et al.*, 1985). The most likely explanation is that this translocational intermediate is embedded in the outer membrane in a proteinaceous environment which is different from the initial protease-sensitive binding site on the mitochondrial surface.

The stage 3 intermediate can be imported into the inner membrane in an energy-dependent manner and can be correctly assembled after protease treatment of mitochondria. Thus, it can be excluded that the pretreatment of mitochondria with low protease concentrations (Fig. 3; Zwizinski *et al.*, 1984; Schmidt *et al.*, 1985) leads to unspecific damage of mitochondria, *e.g.* by generation of peptides which uncouple mitochondria. Our findings confirm that a protease-sensitive component on the mitochondrial surface is indeed required for specific import of proteins.

It cannot be excluded that parts of the protease-resistant precursor (stage 3) are still exposed to the outer surface of the outer membrane but are not cleaved by low concentrations of proteinase K or trypsin. It seems very unlikely, however, that major parts or even the whole precursor are still outside the outer membrane but are resistant against the proteases due to a conformational protease resistance. (i) The precursor in the reticulocyte lysate (stage 1) is very protease sensitive. (ii) Interaction of the precursor with the mitochondrial import machinery does not seem to induce a conformational protease resistance. The major fraction of precursor which was bound in a specific (import-competent) manner at 2 °C (stage 2) was protease sensitive, and the intermediate which was spanning contact sites (stage 4a) was also found to be protease sensitive

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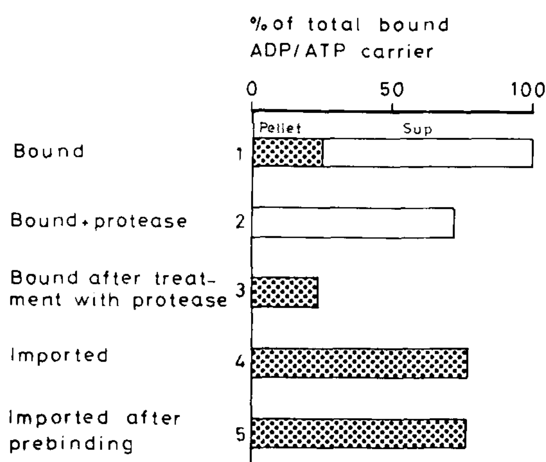

FIG. 5. **The protease-protected bound precursor can be extracted at pH 11.5.** Forty μl of reticulocyte lysate were incubated with mitochondria (20 μg of mitochondrial protein) in the presence of antimycin A and oligomycin (reactions 1–3 and 5) or ascorbate plus TMPD (reaction 4). The reaction mixture contained BSA buffer up to a final volume of 400 μl. The mitochondria of reaction 3 had been pretreated with trypsin (20 μg/ml final concentration) in BSA-containing buffer (Zwizinski *et al.*, 1984). All samples were incubated for 25 min at 25 °C. Sample 2 was treated with trypsin (20 μg/ml final concentration) and sample 4 with proteinase K (200 μg/ml final concentration). Mitochondria were reisolated from all samples. Mitochondria of sample 5 were resuspended in 300 μl of BSA-containing buffer, and ascorbate plus TMPD was added; after 15 min at 25 °C, the sample was treated with proteinase K (200 μg/ml final concentration), and mitochondria were isolated again. Alkaline treatment (Fujiki *et al.*, 1982a, 1982b) of mitochondria was carried out as described (Schleyer and Neupert, 1985) except that the mitochondria were resuspended in 0.1 M Na_2CO_3 at a protein concentration of 0.04 mg/ml and that the incubation was performed for 30 min at 0 °C. Separation of pellets and supernatants (*Sup*) and immunoprecipitation were performed as described (Hartl *et al.*, 1986).

(see below). (iii) After lysis of the mitochondria with Triton X-100, all accumulated precursors as well as the mature protein were sensitive to low protease concentrations (Fig. 1 and data not shown). At very high concentrations of proteinase K (Fig. 1*A*) the outer membrane barrier is degraded.[3] Despite the possibility that the precursor is still partly outside the outer membrane, the data described here are consistent with the existence of a translocational intermediate (stage 3) having the characteristics of a peripheral membrane protein which is beyond the recognition step but before the $\Delta\psi$-dependent translocation.

About 25% of the bound precursor was import incompetent. This unspecifically bound form was protease sensitive but resistant to carbonate extraction. With this species, part of the molecule may interact with the lipid phase of the outer membrane. Generation of this species was dependent on the presence of mitochondria and could not be explained by artifacts such as binding to tube walls. The absolute amount of carbonate-resistant bound precursor was the same with untreated mitochondria and mitochondria pretreated with proteases (Fig. 5, *columns 1* and *3*). When protease-pretreated mitochondria were added to a normal import assay the import competence of the precursor was not affected (Zwizinski *et al.*, 1984). Other precursor proteins which need a membrane potential for import, including subunits IV (cytochrome c_1) and V (FeS protein) of the cytochrome bc_1 complex and subunits 2 and 9 of the F_0F_1-ATPase, are not efficiently imported when first bound to de-energized mitochondria (Pfanner and Neupert, 1985). Most precursor proteins associated with de-energized mitochondria appear to be bound in an import-incompetent manner. For the FeS protein it has also been shown that this unspecifically bound precursor is resistant to carbonate extraction and sensitive to proteases (Hartl *et al.*, 1986).

After Binding to De-energized Mitochondria at Low Temperature, the Major Fraction of Precursors Remains Accessible to External Proteases—Following binding of precursor to de-energized mitochondria at 25 °C, practically all of the specifically bound precursor (stage 3) is beyond the stage requiring the protease-sensitive component on the mitochondrial surface. Can precursor proteins be accumulated at an earlier stage of the import pathway where they are still sensitive to mild protease treatment? Precursor proteins were bound to de-energized mitochondria at low temperature (2 °C). After reisolation, half of the mitochondria were treated with low concentrations of trypsin; the other half remained untreated. The mitochondria were then incubated at 25 °C, and a membrane potential was established to import ADP/ATP carrier to the mature functional form (stage 5). Only about 40% of the imported ADP/ATP carrier originated from the protease-resistant translocational intermediate (stage 3). The major fraction of specifically bound precursors was in a protease-accessible location (stage 2) (Table I).

In a parallel experiment, after binding of precursor at 2 °C, the de-energized mitochondria were reisolated and incubated at 25 °C ("25 °C chase"). Then protease treatment and re-energization at 25 °C were performed as before. By incubation at 25 °C under de-energized conditions, the precursors could be chased from the protease-accessible form (stage 2) to the protease-protected form (stage 3). After re-energization it was then chased to the mature protein (stage 5) (Table I). Thus, two successive steps in the binding of precursor to de-energized mitochondria can be defined.

Precursor to the ADP/ATP Carrier Is Imported via Translocation Contact Sites—Several mitochondrial proteins which are synthesized with amino-terminal presequences have been shown to be imported via translocation contact sites, including subunit 2 of the F_0F_1-ATPase and subunits IV and V of the cytochrome bc_1 complex. At translocation contact sites, outer and inner membranes come close enough together that

TABLE I

Binding at low temperature traps precursors in a protease-accessible location

Eighty μl of reticulocyte lysate containing radiolabeled precursor proteins were incubated with mitochondria (40 μg of mitochondrial protein) in the presence of antimycin A and oligomycin for 20 min at either 25 or 2 °C. Mitochondria were reisolated and resuspended in 600 μl of BSA-containing buffer in the presence of antimycin A and oligomycin. Samples were divided and one half was treated with trypsin in SEM (10 μg/ml final concentration) while the other half received SEM. Ascorbate plus TMPD was then added to all samples. After 15 min at 25 °C, mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography. In a parallel experiment ("25 °C chase"), the reaction mixture was incubated for 15 min at 25 °C prior to dividing samples but otherwise treated as described above. The ratio between the amount of ADP/ATP carrier imported after the protease treatment and the amount of ADP/ATP carrier imported without protease treament was determined.

Binding at	25 °C	2 °C	2 °C + 25 °C chase
[Import after protease treatment] / [Import without protease treatment] =	0.98	0.41	0.96

[3] H. Schwaiger, V. Herzog, and W. Neupert, unpublished data.

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they can be spanned simultaneously by a polypeptide chain (Schleyer and Neupert, 1985; Hartl *et al.*, 1986). It is of particular interest whether precursors without presequences, such as the precursor to the ADP/ATP carrier, are also imported via these contact sites.

In vitro synthesized precursor was incubated with energized mitochondria at low temperature (Fig. 6*B*, *columns 1–3*). Valinomycin, which completely inhibits the import of free precursor under the conditions used (see Fig. 6*B*, *lane 4*; Schleyer *et al.*, 1982; Schleyer and Neupert, 1985; Hartl *et al.*, 1986), was then added. Mitochondria were immediately reisolated from one aliquot of the incubation (reaction 1). A second aliquot was treated with protease at a low concentration which leaves the mitochondrial outer membrane barrier intact (Zwizinski *et al.*, 1984; Schleyer and Neupert, 1985; Hartl *et al.*, 1986), and mitochondria were then reisolated (reaction 2). The major part of precursors associated with these latter mitochondria was sensitive to this protease treatment (Fig. 6*B*, *column 2*) demonstrating that a piece of these precursors was outside the outer membrane. A third aliquot was warmed to 25 °C after the addition of valinomycin, and imported ADP/ATP carrier was determined (Fig. 6*B*, *column 3*). In this case, most of the precursor could be completely imported without further need of a membrane potential. Thus, the energy-dependent interaction of precursor with the inner membrane had already taken place when incubation with energized mitochondria had been performed at 2 °C.

The precursors associated with energized mitochondria at low temperature (stage 4a) extend from outside the outer membrane, where they are sensitive to low concentrations of protease, into the inner membrane where the membrane potential-dependent stage of import occurs. Therefore, outer and inner membranes come close enough together to be spanned by the ADP/ATP carrier polypeptide chain.

In the experiments described in Fig. 7, we examined whether an intermediate could be generated from the stage 3 intermediate which is partially inserted into the inner membrane. De-energized mitochondria were incubated with precursor at 25 °C. After reisolation, the mitochondria were treated with trypsin to leave the protease-protected translocation intermediate in the outer membrane (stage 3). Mitochondria were re-energized by addition of ascorbate plus TMPD at 25 °C (Fig. 7*A*) or at 2 °C (Fig. 7*B*). Valinomycin was then added (samples 1 and 3). The control samples (2 and 4) received valinomycin before the addition of ascorbate plus TMPD; thereby, import was completely inhibited. Samples 3 and 4 were incubated for 15 min further at 25 °C. Finally, imported ADP/ATP carrier (stage 5) was determined in all samples. Fig. 7*B*, *column 1*, shows that by energization at 2 °C only a small fraction of precursor is completely imported into the inner membrane. Warming the mitochondria to 25 °C led to complete import of precursor even in the absence of a membrane potential (Fig. 7*B*, *column 3*). Thus, the $\Delta\psi$-dependent entrance into the inner membrane had already taken place, but the import was not complete when re-energization was performed at 2 °C. A similar result was obtained when re-energization was performed at 0 °C (not shown).

In summary, the translocational intermediate in the outer membrane (stage 3) can be converted to an intermediate which is partially imported into the inner membrane (stage 4). This intermediate can then be chased to the mature protein (stage 5).

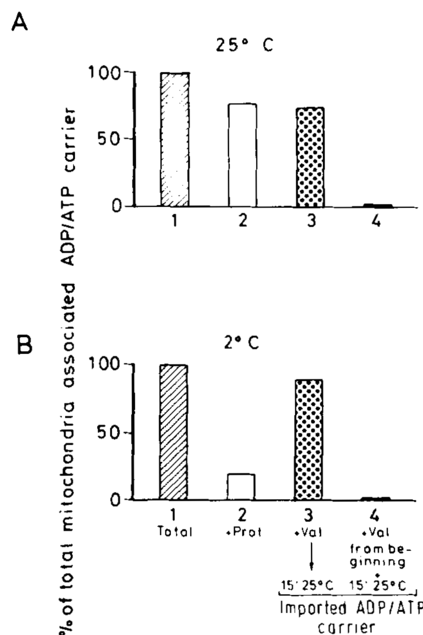

FIG. 6. **The precursor to the ADP/ATP carrier is imported via translocation contact sites.** Reticulocyte lysate, mitochondria, BSA-containing buffer, and ascorbate plus TMPD were incubated for 20 min at 25 °C (*A*) or 2 °C (*B*). Then valinomycin (*Val*) was added. From one aliquot (reaction 1), mitochondria were reisolated immediately. A second aliquot (reaction 2) was treated with proteinase K (10 μg/ml final concentration) (*Prot*), and mitochondria were then reisolated. A third aliquot (reaction 3) was incubated for 15 min at 25 °C, treated with proteinase K (200 μg/ml final concentration), and mitochondria were then reisolated. A fourth aliquot (reaction 4) received valinomycin before the first incubation and was then treated as described for reaction 3. The amounts of the additions were such that each aliquot contained 40 μl of reticulocyte lysate and 20 μg of mitochondrial protein in a final volume of 300 μl. ADP/ATP carrier was immunoprecipitated from all samples.

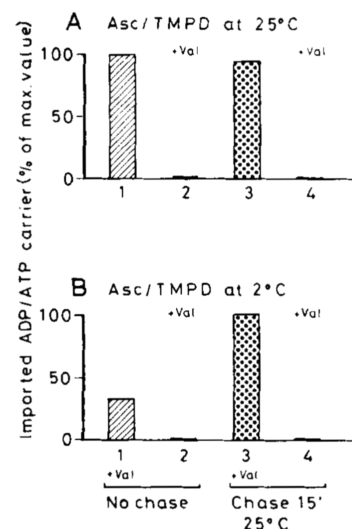

FIG. 7. **The protease-protected bound precursor can be chased to an intermediate which is partially inserted into the inner membrane.** Binding of precursors to de-energized mitochondria, reisolation of mitochondria, resuspension in BSA-containing buffer in the presence of antimycin A and oligomycin, and treatment with trypsin (10 μg/ml final concentration) were performed as described in the legend to Fig. 2*A*. The samples received ascorbate plus TMPD (*Asc/TMPD*) and were incubated for 2 min at 25 °C (samples *A*) or 30 s at 2 °C (samples *B*), followed by the addition of valinomycin (*Val*). Samples *2* and *4* received valinomycin before the addition of ascorbate plus TMPD. Samples *1* and *2* were treated with proteinase K (200 μg/ml final concentration). Samples *3* and *4* were incubated for 15 min at 25 °C and treated with proteinase K at the same concentration. Mitochondria were reisolated, and ADP/ATP carrier was immunoprecipitated.

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The stage 4 intermediate corresponds in several respects to the translocation intermediate (stage 4a) described in Fig. 6 which spans contact sites between outer and inner membranes. Both can be generated during energization of mitochondria at 2 °C and can be chased to the mature protein in the absence of a membrane potential. It should be noted, however, that precursors inserted into the outer membrane (protease-resistant bound precursor, stage 3) never became sensitive to low protease concentrations which leave the outer membrane barrier intact. This was observed with de-energized and energized mitochondria at different temperatures (not shown).

Cytosolic Cofactors for the Import of Mitochondrial Precursor Proteins—Several studies on cytosolic cofactors for import of mitochondrial precursor proteins have been published (Argan *et al.*, 1983; Miura *et al.*, 1983; Firgaira *et al.*, 1984; Ohta and Schatz, 1984; Argan and Shore, 1985; Burns and Lewin, 1986). We have examined whether a requirement for such factors exists in the case of the ADP/ATP carrier and at which step they may be required.

Reticulocyte lysate containing ^{35}S-labeled precursor proteins ("^{35}S-lysate") was passed over Sephadex G-25. The pass-through fraction was incubated with energized *Neurospora* mitochondria. The mitochondria had been isolated by two different methods: at low salt concentrations ("SEM mitochondria") (Fig. 8*A*, *lanes 4–6*) or in the presence of 180 mM KCl including three washes in 180 mM KCl ("KCl mitochondria") (Fig. 8*A*, *lanes 1–3*). When untreated reticulocyte lysate not containing *Neurospora* precursor proteins ("no synthesis lysate") was mixed to the reaction mixture before the addition of the mitochondria, the import of ADP/ATP carrier was normal for both mitochondrial preparations (Fig. 8*A*, *lanes 1* and *4*). When BSA-containing buffer was added instead of the no synthesis lysate, import of the ADP/ATP carrier into the SEM mitochondria was still normal (Fig. 8*A*, *lanes 5* and *6*), but import into the KCl mitochondria was inhibited (Fig. 8*A*, *lanes 2* and *3*). Similar results were obtained for the import of subunits 2 and 9 of the F_oF_1-ATPase (not shown). These results suggest that a component ("cytosolic cofactor") which is present in rabbit reticulocyte lysate and which binds to *Neurospora* mitochondria is necessary for the import of several mitochondrial precursor proteins.

As shown above (Fig. 5), two different forms of precursors bound to de-energized mitochondria at 25 °C can be defined. Specifically bound precursor (stage 3) can be extracted at alkaline pH, whereas unspecifically bound precursor is resistant to carbonate extraction. Precursor proteins were incubated with de-energized KCl mitochondria (Fig. 8*B*, *columns 2* and *3*) under conditions where import into energized mitochondria was inhibited (*cf.* Fig. 8*A*, *lanes 2* and *3*). All of the bound precursor was resistant to extraction by carbonate. When no synthesis lysate was included, the normal distribution of carbonate-soluble and carbonate-resistant bound precursors was observed (*cf.* Fig. 8*B*, *column 1*, and Fig. 5, *column 1*). Thus, the inhibition of import in the absence of this "cofactor" can be accounted for at the level of binding of precursor to de-energized mitochondria. We suggest that the cofactor component is required for the specific interaction of precursor with mitochondria. It should be emphasized that the total amount of precursor associated with de-energized mitochondria does not give any information as to the specificity of binding. As shown in Fig. 8*B*, *column 3*, the total amount of binding in buffer with 200 mM KCl was close to the control value, but the binding was completely unspecific.

In summary, passage of reticulocyte lysate over Sephadex G-25 leads to an inhibition of specific binding of precursor to salt-washed mitochondria. This inhibition can be prevented by a component which is present in reticulocyte lysate and which can bind to *Neurospora* mitochondria (cytosolic cofactor). The nature of this cofactor, however, remains elusive so far. A number of observations in this context awaits explanation, such as the finding that import into mitochondria which had been incubated with filtered lysate was irreversibly blocked when the mitochondria were subsequently incubated with untreated lysate.[1]

Pretreatment of reticulocyte lysate containing ^{35}S-labeled precursor proteins with high concentrations of RNase A at elevated temperature or in the presence of chelating agents has been reported to inhibit the import of several mitochondrial precursor proteins (Firgaira *et al.*, 1984). When we pretreated reticulocyte lysate with RNase A, the import of ADP/ATP carrier into mitochondria was still normal, but the import of the subunit 2 of the F_oF_1-ATPase ($F_1\beta$) was inhibited.[1] The RNase-sensitive component in the reticulocyte lysate could be a ribonucleoprotein complex as suggested by Firgaira *et al.* (1984). At present, however, it cannot be ex-

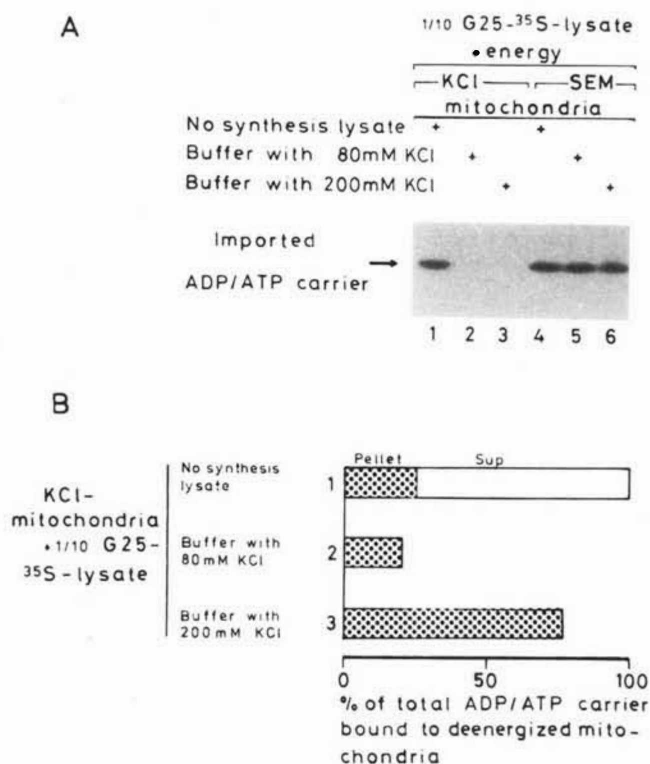

FIG. 8. **Passage of reticulocyte lysate over Sephadex G-25 inhibits the import and the specific binding of ADP/ATP carrier to salt-washed mitochondria.** *A*, *G25-^{35}S-lysate* (see "Experimental Procedures") (24 µl) was incubated with isolated mitochondria (20 µg of mitochondrial protein) in the presence of ascorbate plus TMPD in a final volume of 240 µl. The mitochondria had been isolated by differential centrifugation either in SEM (see "Experimental Procedures") or in KM (180 mM KCl, 10 mM MOPS, adjusted to pH 7.2 with KOH) including three washes in KM and resuspension in KM. Samples *1* and *4* contained no synthesis lysate; samples *2* and *5* the BSA-containing buffer described under "Experimental Procedures," and samples *3* and *6* BSA buffer with 200 mM KCl (instead of 80 mM KCl and 250 mM sucrose). Incubation was performed for 25 min at 25 °C. Mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography. Experiment *B* was performed as described for *A* (reactions 1–3) with the following modifications. Antimycin A and oligomycin were added instead of ascorbate plus TMPD. Alkaline treatment of mitochondria was performed as described in the legend to Fig. 5, and ADP/ATP carrier was immunoprecipitated.

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cluded that treatment with excessive RNase concentrations affects a component other than RNA.

DISCUSSION

We describe here the trapping of distinct translocational intermediates on the import pathway of the ADP/ATP carrier into mitochondria. The interpretation of our data is summarized in the following model (Fig. 9). After synthesis on cytoplasmic polysomes, the ADP/ATP carrier precursor protein is transported in a water-soluble form (stage 1) to mitochondria (Zimmermann and Neupert, 1980). The precursor interacts with a receptor-type protein on the mitochondrial surface which is sensitive to protease treatment (stage 2). The precursor is then directed to saturable protease-protected sites in the outer membrane (stage 3). This step is rather slow at low temperature (2 °C). The stage 3 intermediate can be extracted at alkaline pH and seems to be associated with proteinaceous components in the outer membrane which may represent as yet unknown protein(s) or a part of the protease-sensitive receptor on the mitochondrial surface. In the latter case this part would be different from the site which initially reacts with the precursor at stage 2. The membrane potential-dependent entrance into the inner membrane (stage 4) occurs at contact sites between outer and inner membranes even at low temperature. Finally, the ADP/ATP carrier is completely transported into the inner membrane and assembled to the functional dimer (stage 5). This step can occur in the absence of a membrane potential but is impeded at lower temperatures.

When free precursor was incubated with energized mitochondria at low temperature, a translocational intermediate reaching from the outside of the outer membrane into the inner membrane was produced (stage 4a). This apparent divergence of the import pathway would suggest that the sequence of the various import reactions is not strictly fixed in all cases.

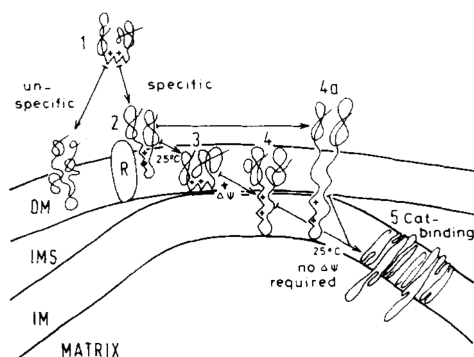

FIG. 9. **Model for the translocation of ADP/ATP carrier into mitochondria.** After synthesis on cytosolic polysomes, the precursor to the ADP/ATP carrier is released in a water-soluble form into the cytosol (stage *1*). It binds to a receptor protein (*R*) on the mitochondrial surface (stage *2*). The precursor is then directed to saturable and proteinaceous sites in the outer membrane (*OM*) where it is protected against proteases (stage *3*). The entrance into the inner membrane (*IM*) occurs at translocation contact sites and requires the membrane potential ($\Delta\psi$) (stage 4). The completion of translocation into the inner membrane and the assembly to the functional dimer which is able to bind carboxyatractyloside (*Cat*) (stage *5*) can occur in the absence of $\Delta\psi$. Incubation of precursor with energized mitochondria at low temperature leads to an intermediate which reaches from the outside of the outer membrane into the inner membrane (stage *4a*). This intermediate can also be chased to the functional form (stage *5*) in the absence of $\Delta\psi$. The specific binding of precursor (stage *2* and/or stage *3*) seems to require a cytosolic cofactor. Unspecifically bound precursor interacts with the lipid phase of the outer membrane, but part of the precursor is still accessible to externally added proteases. *IMS*, intermembrane space.

We conclude that at least four different steps can be defined in the import of ADP/ATP carrier: (i) interaction with a protease-sensitive component on the mitochondrial surface; (ii) translocation of major parts of the precursor into or across the outer membrane by a step which is slow at lower temperature; (iii) membrane potential-dependent insertion into the inner membrane; (iv) translocation of the entire precursor into the inner membrane (which is also slower at low temperature) and assembly to the dimeric form. The order of steps (ii) and (iii) is not fixed. It can be as described above (Fig. 7), or step (iii) occurs before step (ii) (see Fig. 6).

What are the components necessary for the import of mitochondrial precursor proteins? First, a cytosolic activity which is present in reticulocyte lysate and which binds to *Neurospora* mitochondria (cytosolic cofactor) may be necessary for the specific binding of precursor to mitochondria. It is not known how many precursor molecules of the ADP/ATP carrier are present in the cytosolic high molecular weight aggregates (Zimmermann and Neupert, 1980) and whether other precursor proteins and cytosolic cofactors are included in these aggregates. Second, a protease-sensitive component on the mitochondrial surface with many characteristics of a receptor is required at an early phase of import (stage 2). Further studies, including Scatchard analysis at low temperature, competition experiments, and isolation of the protein(s) are required to clarify whether this component is a receptor according to the classical definition (Kahn, 1975). A translocational intermediate (stage 3) is anchored to the outer membrane by saturable and probably proteinaceous sites. Preliminary data show that the translocational intermediates in contact sites (stage 4a and stage 4) are also accessible to aqueous perturbants.[1] It cannot be excluded that this is only caused by a local disturbance of the lipid bilayer. It seems more likely, however, that these intermediates are shielded from the hydrophobic core of the membranes by proteinaceous components.

We propose a model where proteins play important roles in the import of the ADP/ATP carrier into mitochondria. At present, it is not known whether the proteins of the import machinery form one complex which contains receptors as well as a "transport pore" or whether these components are separated in the lipid phase. On the other hand, it is very unlikely that lipids alone represent the import apparatus for the translocation of the ADP/ATP carrier across the mitochondrial membranes. Other models have emphasized a mainly lipophilic environment for mitochondrial protein import (Roise *et al.*, 1986; von Heijne, 1986; Hurt and van Loon, 1986). It should be noted that under our conditions about 25% of the precursor was bound to the hydrophobic core of the outer membrane. This binding was also found with protease-pretreated (and therefore import-incompetent) mitochondria. Quantitative analysis showed that in untreated mitochondria this bound precursor was also not imported. It cannot be excluded, however, that a very minor fraction of this form of precursor is eventually imported. For example, the initial interaction of precursor with mitochondria could occur with lipids of the outer membrane. By binding to protein components of the outer membrane, the ratio between free and mitochondria-associated precursor would be shifted to the latter case. The described translocation reactions might then take place.

Interestingly, for the translocation of proteins across the membrane of the endoplasmic reticulum a proteinaceous pore has been recently proposed (Gilmore and Blobel, 1985; Evans *et al.*, 1986).

By energization of mitochondria for a very short time (30

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s) at 0 °C, the translocational intermediate in the outer membrane (stage 3) can be transported to the stage 4 intermediate which is partially inserted into the inner membrane. Thus, it is unlikely that contact sites between outer and inner membranes must be formed by the action of the membrane potential and that the stage 3 precursor has to diffuse laterally in the lipid phase of the outer membrane to reach contact sites. Independent evidence suggests that those parts of the outer membrane which are in close proximity to the contact regions are required for the import of the ADP/ATP carrier.[3] In summary, we propose that proteins of and nearby the contact sites are essential parts of the mitochondrial translocation apparatus.

The question arises as to how the import pathway of the ADP/ATP carrier is related to the import pathways of other precursor proteins. The import of most precursor proteins, including those to the outer membrane protein porin, subunits IV and V of the bc_1 complex, and subunits 2 and 9 of the F_0F_1-ATPase, are inhibited by pretreatment of mitochondria with low protease concentrations (Zwizinski *et al.*, 1984; Schmidt *et al.*, 1985; Hartl *et al.*, 1986). A water-soluble form of porin, with properties of the porin precursor (Pfaller *et al.*, 1985), competes for the generation of the translocational intermediate in the outer membrane (stage 3). Furthermore, a protease-protected translocational intermediate of porin has been observed which is located past the initial interaction of the precursor with a protease-sensitive component on the mitochondrial surface.[4] This suggests that the import apparatus of the ADP/ATP carrier shares a component with the import machinery for porin at the level of stage 2 and/or stage 3.

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