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Characterization of the Initial Steps of Precursor Import into Rat Liver Mitoplasts[1]

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Mitochondria have two independent protein-import machineries, one in the outer membrane (the Tom system) and the other in the inner membrane (the Tim system). Here, we have characterized the initial steps of precursor import into rat liver mitoplasts. The import reaction was separated into two stages, consisting of precursor binding to the mitoplasts at 0-10°C, and a subsequent chase reaction at 30°C. This assay revealed four distinct precursor-import steps: $\Delta\Psi$-dependent initial binding of the precursor, precursor transfer to the Tim23-Tim17 stage, $\Delta\Psi$-dependent translocation of the presequence across the inner membrane, and the complete translocation of the mature portion of the precursor. Antibodies against the intermembrane space domain of Tim23 inhibited neither the precursor binding nor the subsequent translocation of the presequence across the inner membrane. In contrast, the antibodies inhibited the complete translocation of the mature domain of the precursor across the inner membrane. Immunoprecipitation with anti-Tim23 IgGs revealed that the precursor-Tim23 complex increased with time and temperature after the initial targeting of the precursor to the mitoplasts. These results suggest that the precursor is first targeted to the inner membrane component $\Delta\Psi$-dependently, then transferred to the Tim system consisting of Tim23-Tim17, and finally imported into the matrix.

Key words: inner mitochondrial membrane, mitochondria, precursor, protein import, Tim proteins.

Most mitochondrial proteins are synthesized in the cytosol as precursors with basic, amphiphilic mitochondria-targeting signals (MTS) at the amino-terminus (*1-5*). Cytoplasmic chaperones such as hsp70 and mitochondrial import stimulation factor (MSF) bind the precursors and target them to the import receptors of the mitochondrial surface. MSF (or a similar factor in yeast cytosol) delivers the bound precursors to the Tom70/Tom37 complex in an ATP-dependent manner (*5-8*). The precursor is then transferred to the Tom20/Tom22 complex and imported into the mitochondria through two distinct import channels in the outer (Tom system) and the inner membranes (Tim system). Hsp70 delivers the bound precursors directly to the Tom20/Tom22 complex in the reaction which does not require ATP hydrolysis. In yeast, the Tom system is composed of at least 9 proteins: Tom5, Tom6, Tom7, and Tom40, which form an import channel; and Tom72, Tom70, Tom37, Tom22, and Tom20, which function as import receptors. To date, 10 proteins have been identified as members of the Tim system in yeast: Tim10, Tim11, Tim12, Tim14, Tim17, Tim22, Tim23, Tim33, Tim44, and Tim54 (*1-5*). Tim17 and Tim23, two structurally related multi-spanning membrane proteins, are essential components and form a complex which probably constitutes the preprotein-conducting channel (*9-12*). Tim23 is reported to form dimers in response to $\Delta\Psi$, which is supposed to function as a precursor receptor on the surface of the inner membrane, and the binding of the presequence induces a dissociation of Tim23 dimers and also triggers an opening of the precursor-conducting channel (*13*). Tim44, a peripheral protein bound to the matrix side of the inner membrane, collaborates with mhsp70 to drive the complete translocation of the precursor proteins into the matrix (*14-16*). On the other hand the inner membrane proteins, Tim22, Tim54, and the intermembrane space proteins Tim10 and Tim12 act as an import machinery for the metabolite carrier proteins of the inner membrane, such as the ADP-ATP carrier (AAC), phosphate carrier, and dicarboxylate carrier (*17-20*). The involvement of Tim22 in the integration of Tim23 into the inner membrane has been recently reported (*21*). The Tom and Tim precursor-

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Abbreviations: AAC, ADP-ATP carrier; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX IV, cytochrome *c* oxidase subunit IV; DHFR, dihydrofolate reductase; mhsp70, mitochondrial hsp70; MPP, mitochondrial matrix-processing peptidase; MSF, mitochondrial import-stimulation factor; MTS, mitochondria-targeting signals; MTX, methotrexate; pAd, preadrenodoxin; Tim and Tom, translocase of inner and outer mitochondrial membrane.

translocation systems appear to be highly conserved among species; Tom20 (*22-26*), Tim17, Tim23, and Tim44 (*27-29*) have been reported for mammalian mitochondria, which also, however, contain several unique components such as Tom34 (*30*), metaxin (*31*), and OM37 (*32*).

The mechanism of mitochondrial protein import has been investigated extensively with yeast and fungi but has yet to be sufficiently studied in the mammalian system. In addition, the way in which the precursor proteins that are translocating the Tom channel are engaged by the Tim machinery, and the way in which $\Delta\Psi$ functions in the subsequent precursor translocation are less well understood. Here, we analyzed the initial steps of precursor targeting and translocation across the inner membrane with rat liver mitoplasts. A two-stage assay for the import reaction, precursor binding at 0-10°C and the subsequent chase at 30°C, revealed several intermediate steps involved in the precursor translocation across the inner membrane. At 0-10°C, pAdDHFR that was initially targeted to the inner membrane moved slowly to the Tim23-Tim17 stage, where the presequence of a small fraction of the bound precursor was translocated across the inner membrane and processed by mitochondrial processing peptidase (MPP) on the matrix side, while a mature segment remained exposed to the exterior of mitoplasts. Upon shifting the reaction up to 30°C under the import-arrested condition with methotrexate (MTX), the presequence portion was translocated and processed, whereas the mature portion still remained associated with Tim23-Tim17. In the absence of MTX, on the other hand, the mature portion was released from Tim23-Tim17 and completely translocated across the inner membrane, becoming resistant to the externally added protease. Having established the assay condition for the initial steps, we examined the effect of CCCP, a synthetic mitochondria-targeting signal peptide, salt, and antibodies against Tim23 or Tim17. These experiments revealed the following. (i) The initial binding of a precursor to the inner membrane occurred through MTS in a $\Delta\Psi$-dependent manner, and was followed by the association with Tim23. The initial binding of the precursor and its subsequent transfer to the Tim23-Tim17 stage were not inhibited by anti-Tim23 IgGs, (ii) The subsequent translocation of the presequence portion across the inner membrane was also dependent upon $\Delta\Psi$, blocked by the synthetic signal peptide, but was not inhibited by anti-Tim23 antibodies, (iii) The complete translocation of the mature portion of partially translocated precursor across the inner membrane did not require $\Delta\Psi$, but was inhibited by anti-Tim23 IgGs. Two $\Delta\Psi$-dependent and signal peptide-recognizing steps were thus revealed. In marked contrast, the binding of AAC to the inner membrane did not require $\Delta\Psi$, but the subsequent membrane integration did. Therefore, similar to the yeast system, the import of AAC into the inner membrane is mediated by a protein-translocation machinery distinct from the Tim23/Tim17 system in mammalian mitochondria.

MATERIALS AND METHODS

Materials—The antibodies against Tom20 (*25*), Tim17, Tim23, Tim44, and subunit IV of cytochrome *c* oxidase (COX IV) from rat liver mitochondria were prepared as described (*28*). The peptides SCC(1-19) and SCC(1-19)M were synthesized described in Ref. *8*. The antibodies against the N-terminal intermembrane space domain of rat Tim23 were affinity-selected from the IgGs against rat Tim23 using the recombinant protein of rat Tim23 (residues 1-75) fused to the C-terminal of glutathione S-transferase. Mutant adrenodoxin precursors carrying several deletions in the presequence portion were expressed in an *in vitro* transcription and translation system as previously described (*33*). The cDNA encoding *Neurospora crassa* ADP-ATP carrier (AAC) was obtained from R. Lill.

Preparation of Rat Liver Mitochondria and Mitoplasts—The preparation of mitochondria from rat liver cells was performed as described (*34*). Mitoplasts were prepared as follows. Mitochondria were incubated at 1 mg/ml with various concentrations of TPCK-treated trypsin (Sigma) in the homogenization buffer (10 mM HEPES-KOH, pH 7.4, containing 0.22 M mannitol, and 0.07 M sucrose) at 0°C for 30 min, then incubated with 10 mg/ml soybean trypsin inhibitor (Sigma) at 0°C for 30 min. These treated mitochondria were diluted into 9 volumes of 10 mM HEPES-KOH buffer (pH 7.4) ("hypotonic buffer") containing protease inhibitor mix (100 μg/ml trypsin inhibitor and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A) and incubated at 0°C for 30 min. The reaction mixture was then centrifuged in a microfuge at 6,000 rpm for 5 min to isolate the mitoplasts.

Preparation of cDNA for pAdDHFR—The cDNA fragment encoding pAd was prepared by the polymerase chain reaction (PCR) using pSP64-pAd (*34*) as the template and the following oligonucleotides as the primers: 5′CGCCTTCCGGAATTCCCC-GACAGGCT3′ and 5′TATTCTATCCTCGA-GGAGTTCATG3′, where underlining indicates the *Eco*RI site and the *Xho*I site, respectively. The PCR fragment thus obtained was digested with *Eco*RI and *Xho*I and was cloned into the *Eco*RI-*Hin*dIII sites of pSP65 together with the *Xho*I-*Hin*dIII fragment of mouse DHFR, which had been prepared from pDS5/2-1-COXIV-DHFR (*35*), to obtain pSP-pAdDHFR. The cDNA thus prepared codes for pAdDHFR fusion protein, where the C-terminal 3 amino acid residues of pAd are replaced by the Arg-Ser-Gly-Ile sequence and followed by the entire DHFR sequence. [^{35}S]-pAdDHFR was synthesized in the transcription-translation coupled system with reticulocyte lysate (*34*).

Protein Import into Mitochondria or Mitoplasts—The import of precursor into the mitochondria or mitoplasts was performed as follows. A suspension of 20 μg of mitoplasts or mitochondria in 50 μl of the homogenization buffer containing 1 mM ATP, 20 mM sodium succinate, 5 mM NADH, protease inhibitor mix, 600 μg/ml cytochrome *c*, and 1 mg/ml fatty acid-free BSA ("import buffer") was incubated with the *in vitro* synthesized precursor (pAd, pAdDHFR, or AAC) at 30°C for 10 min or 30 min. After import, the mitochondria or mitoplasts were recovered by centrifugation in a microfuge at 7,000 rpm for 5 min and washed once with the homogenization buffer. If necessary, after import, the reaction mixtures were incubated with 100 μg/ml of proteinase K at 0°C for 30 min, then the mitochondria or mitoplasts were isolated by centrifugation, precipitated with 10% TCA, and analyzed by SDS-PAGE. The precursor binding to the mitoplasts and the subsequent chase reactions were performed as follows. A suspension of 20 μg of mitoplasts in 50 μl of the import buffer was

incubated with *in vitro* synthesized precursors (pAdDHFR or AAC) at 0-10°C for 5 to 30 min ("bind reaction"). After the reaction, the mitoplasts were isolated by centrifugation and, if necessary, washed once with the homogenization buffer. The mitoplasts were resuspended in the import buffer, incubated at 30°C for a further 30 min, then treated with proteinase K at 0°C for 30 min ("chase reaction"). The effect of antibodies against rat Tim17, rat Tim23, or rat Tim44 on the preprotein import or on its bind-and-chase reaction was examined as follows. The mitoplasts were incubated with the antibodies in the import buffer at 0°C for 30 min, washed once with the homogenization buffer, suspended in the import buffer, then subjected to the import assays.

Analysis of Interactions between the Precursor and the Tim Components by Immunoprecipitation—Immunoprecipitation was performed essentially as described previously (*28*). Briefly, after the binding of pAdDHFR or its import into mitoplasts, the mitoplasts were isolated and solubilized with 0.25% digitonin in hypotonic buffer. The solubilized supernatant was subjected to immunoprecipitation with antibodies against rTim17 or rTim23. The immunocomplexes were recovered by Protein A-Sepharose and subjected to SDS-PAGE, and the gels were analyzed and quantified with a bioimage analyzer FLA2000 (Fuji Film).

RESULTS

Import of Precursor Proteins into Rat Liver Mitoplasts—It is well established that the Tom and Tim translocation systems function independently in yeast mitochondria (*1-5, 36*). We first confirmed that this is also the case with rat liver mitochondria. As shown in Fig. 1A, rat liver mitochondria lost 70-90% of the import activity of preadrenodoxin (pAd) after trypsin treatment at various concentrations. Tom20, the major import receptor of mitochondria, where mitochondria-targeted precursors converge before being transported into the mitochondria (*1-6*), was shown to be degraded by trypsin treatment at a concentration as low as 5 μg/ml (Fig. 1A, lanes 1 through 5). However, when the trypsin-treated and import-inactivated mitochondria were subjected to hypotonic treatment, the mitoplasts acquired even a higher import activity than that of the intact mitochondria (lane 6). Sulfite oxidase, the intermembrane space enzyme, was released to the soluble fraction by this treatment. However, no significant loss was observed for malate dehydrogenase, the matrix-localizing enzyme (data not shown). These results indicate that the observed import did occur through the Tim system and was independent of the import receptors of the outer membrane, confirming

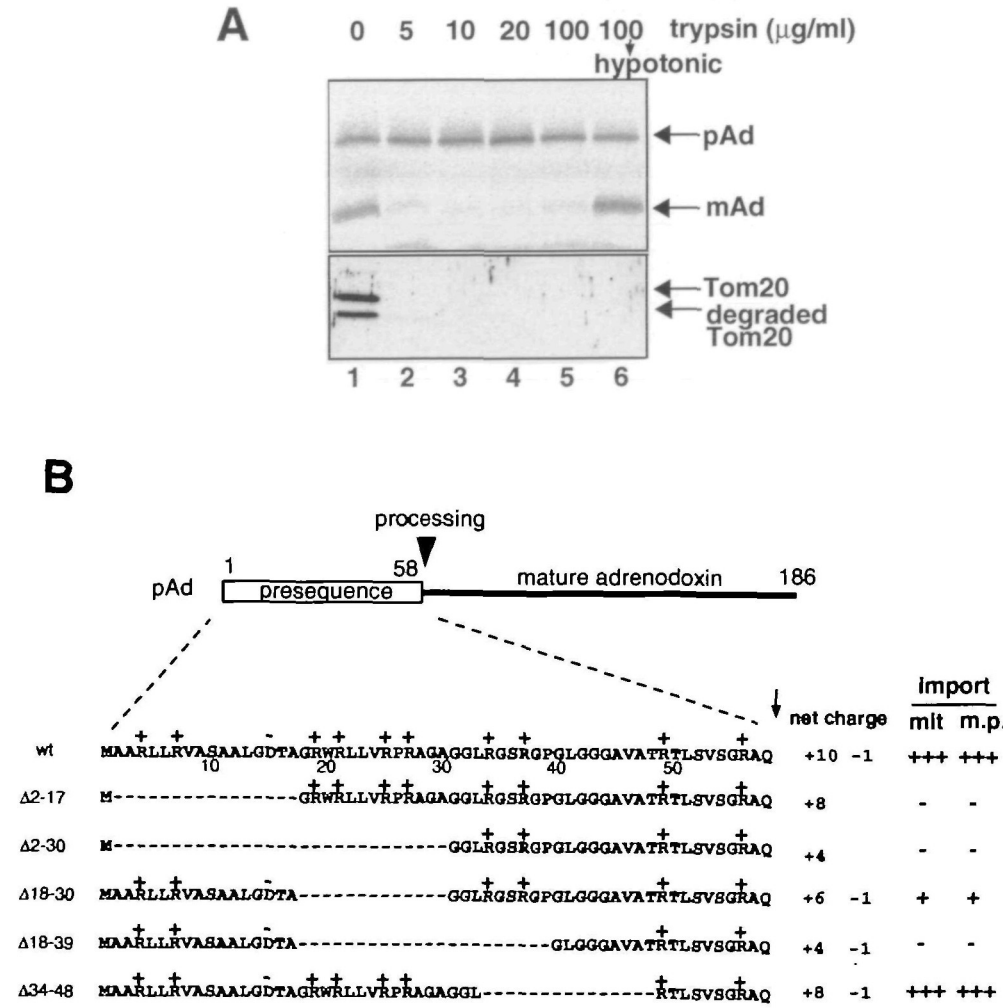

Fig. 1. **Import of pAd and the mutated proteins into rat liver mitoplasts.** (A) Rat liver mitochondria were treated with the indicated concentrations of trypsin (lanes 1-5), or after the trypsin treatment the reaction mixture was subjected to hypotonic treatment to prepare mitoplasts (lane 6). [^{35}S]-pAd or a mutant form synthesized in reticulocyte lysate was imported into the mitochondria or the mitoplasts as described in "MATERIALS AND METHODS." After import, the mitochondria were isolated by centrifugation, subjected to SDS-PAGE and then the gels were analyzed by FLA2000. An aliquot of the mitochondria or the mitoplasts was resolved by SDS-PAGE and then subjected to Western blotting with anti-Tom20 IgG. (B) [^{35}S]-pAd and the deletion mutants were synthesized in the wheat germ lysate system, treated with 7 M urea, then subjected to import into either mitochondria (mit) or mitoplasts (m.p.). The relative import efficiencies: (+++), 50-100% of the activity for pAd; (+), 0-25%; (−), 0%. Since mRNAs for the mutant adrenodoxin precursors were inefficiently translated in the reticulocyte lysate system, they were translated in the wheat germ system. The translation products were dissolved in 7 M urea and used for the import assay, thus circumventing the cytoplasmic chaperone-dependent unfolding step.

the results of yeast mitochondria (*36*). The initial rate of precursor import into the mitoplasts was about twofold higher than that of the intact mitochondria (data not shown), suggesting that the overall reaction of precursor import into the mitochondria is limited at the step of the translocation across the outer mitochondrial membrane.

We next compared the precursor recognition specificity of mitoplasts with that of mitochondria using pAd and mutant proteins in which various regions of the presequence had been deleted (*33*). As summarized in Fig. 1B, no significant differences were apparent between the mitochondria and mitoplasts with respect to the import efficiency of the precursors; the N-terminal region of 2-17 residues and the following 31-38 region of pAd were essential for both import reactions. These results and the competition experiments with a synthetic mitochondria-targeting signal peptide as described below indicate that the recognition specificity of the Tim system for the matrix-targeting signals is similar to that of the Tom system.

Separation of the Overall Import into Precursor-Binding and Subsequent Chase Reactions—As a first step toward analyzing the early steps of precursor import into mitoplasts in more detail, we established an assay in which the reactions of precursor binding and the subsequent import could be measured separately. For this purpose we used a fusion precursor, pAdDHFR, in which pAd was fused to the N-terminus of dihydrofolate reductase (DHFR), so that the import intermediate could be detected by the addition of methotrexate (MTX) to induce the folding of DHFR and to arrest the import at the folded DHFR moiety (see Fig. 4B). When reticulocyte lysate-synthesized pAdDHFR was incubated with the mitoplasts at 10°C for 15 min and centrifuged, pAdDHFR and a small amount of a processed form (mAdDHFR) were recovered from the mitoplasts, both of them being sensitive to proteinase K treatment (Fig. 2, lanes 2 and 3). Thus, most pAdDHFR remains bound to the outer surface of the mitoplasts at 10°C and the presequence

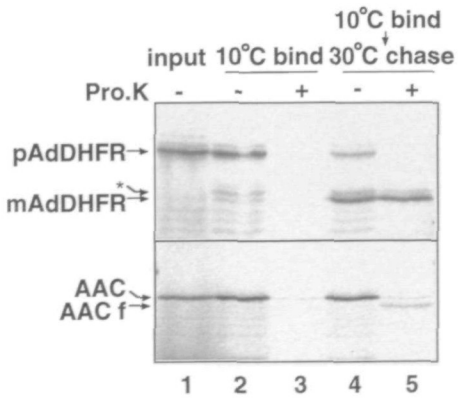

Fig. 2. **Binding and the subsequent import reactions of pAdDHFR and AAC into mitoplasts.** Reticulocyte lysate-synthesized [^{35}S]-pAdDHFR or [^{35}S]-AAC was incubated at 10°C for 15 min with the mitoplasts. The reaction mixtures were centrifuged to isolate the mitoplasts (lanes 2-5). The isolated mitoplasts were resuspended in the import buffer and incubated at 30°C for 30 min (lanes 4 and 5). After the binding or chase reaction, the mixtures were treated with or without 100 μg/ml proteinase K at 0°C for 30 min (lanes 3 and 5). The reaction mixtures were resolved by SDS-PAGE and the gels were analyzed by FLA2000. Input: 50% of pAdDHFR or 20% of AAC used in the import reaction (lane 1).

portion of a small fraction of the bound precursor had crossed the inner membrane to be processed by mitochondrial matrix processing peptidase (MPP). When these mitoplasts were subjected to the chase reaction at 30°C for 30 min, the precursor was processed, and the processed form became resistant to the externally added proteinase K (lanes 4 and 5), indicating that the binding reaction at 10°C was productive and most of the bound precursor was competent for translocation across the inner membrane in the chase reaction. The productive binding of the precursor was also observed at 0°C (see Fig. 5). It should be noted that pAdDHFR underwent two steps of processing during the import: the higher molecular weight intermediate (indicated by an asterisk in the figure) was the major product in the binding reaction, which then underwent further processing to form a lower molecular weight species (indicated by mAdDHFR) during the chase reaction. MPP appears to catalyze the former processing to form the intermediate, whereas mitochondrial intermediate peptidase (MIP) catalyzes the latter and removes an octapeptide from the intermediate to form mature AdDHFR (*33, 37*). A rough calculation indicated that 45% of the precursor in the reaction mixture was recovered from the mitoplasts and 83% of the bound precursor was processed and imported into the matrix.

When reticulocyte lysate-synthesized fungal AAC was subjected to the same reaction as described above, 33% of total added AAC precursor was recovered from the mitoplasts as a proteinase K-sensitive form after the binding reaction (Fig. 2, lanes 2 and 3). It should be noted that rat liver mitochondria behaved similarly to fungal mitochondria in importing fungal AAC; the AAC import depended upon $\Delta\Psi$ and the imported AAC became resistant to both proteinase K digestion and alkali-extraction (data not shown). Judging from the formation of a proteinase K-resistant fragment, 12.5% of the bound precursor was correctly inserted into the inner membrane after the chase reaction (AACf, lanes 4 and 5) (*17, 19, 20*). When $\Delta\Psi$ was dissipated by CCCP during the chase reaction, AACf was not detectable (see Fig. 8B, line 5). These observations confirmed the results with yeast or fungal mitochondria that the insertion of AAC into the inner membrane depends upon $\Delta\Psi$ across the inner membrane (*17, 19, 20*).

pAdDHFR Is Targeted to the Inner Membrane before Moving to the Tim23-Tim17 Stage—We have already shown with rat liver mitochondria that Tim23 and Tim17 form a complex, in which both components are closely apposed to the translocating polypeptide (*28*). To probe the initial steps of precursor import into mitoplasts, we first examined the effects of antibodies against Tim17, Tim23, or Tim44 on the overall import reaction of pAdDHFR. As shown in Fig. 3, anti-Tim17 or Tim23 IgGs inhibited only marginally the processing of pAdDHFR to form mature AdDHFR (w/o Pro.K, lanes 1 through 5), but they inhibited the overall import reaction to a significant extent (Pro.K, lanes 1 through 5). These results suggest that the antibodies against Tim17 and Tim23 inhibit the translocation step of the mature segment rather than the initial steps of precursor binding or the subsequent presequence translocation (see also Figs. 4A and 9). No inhibition was observed with preimmune IgGs or IgGs against Tim44, which is located in the matrix in both a soluble form and an

inner membrane-bound form (lanes 6 through 9) (*28*). Anti-Tim17 or Tim23 IgGs did not inhibit the import of AAC precursor into the inner membrane (lanes 1 through 9, AAC), confirming the report with yeast mitochondria that the import of AAC into the inner membrane depends on the Tim22 system but not on the Tim17-Tim23 system (*17, 19, 20*).

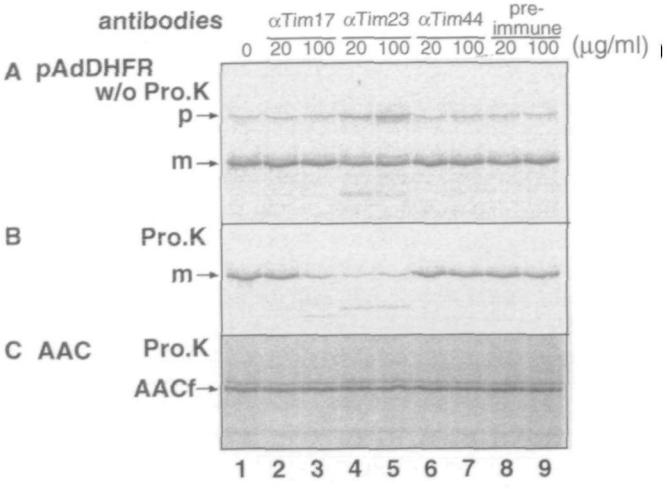

Fig. 3. **Antibodies against Tim17 or Tim23 inhibit translocation of mature portion of pAdDHFR.** Mitoplasts were treated with the indicated concentrations of antibodies at 0°C for 30 min. The mitoplasts were reisolated by centrifugation and used for the import of either reticulocyte lysate-synthesized [^{35}S]-pAdDHFR (panels A and B) or [^{35}S]-AAC (panel C). Where indicated, the reaction mixtures were untreated (w/o Pro.K, panel A) or treated with proteinase K at 0°C for 30 min (Pro.K, panels B and C). AACf: the proteinase K-resistant fragment produced from AAC correctly inserted into the mitochondrial inner membrane. All other conditions are described in "MATERIALS AND METHODS."

We then examined the effect of antibodies against Tim23 on the precursor-binding and the subsequent translocation reactions. As shown in Fig. 4A, the antibodies against Tim23 did not inhibit the binding of pAdDHFR to the mitoplasts (lanes 1 through 4). In contrast, when the pAdDHFR-loaded mitoplasts were incubated with the same antibodies before the chase reaction, the import of pre-bound pAdDHFR was almost completely blocked (lanes 6 and 7), whereas only a marginal inhibition was observed with antibodies against COX IV (compare lanes 5 and 8), confirming the finding that anti-Tim23 IgGs inhibit the membrane-transport process but not the targeting process (see Figs. 3 and 9). The same results were obtained with IgGs against the N-terminal intermembrane space domain of rTim23 (data not shown). These observations suggest that the precursor protein is targeted first to a component distinct from Tim17 and Tim23, and then transported into the matrix *via* Tim17 and Tim23 proteins. We examined this possibility by immunoprecipitation of the import-arrested pAdDHFR with antibodies against rat Tim17 or rat Tim23. As shown in Fig. 4B, the proteinase K-protection assay indicated that a significant fraction of the translocating polypeptide was arrested as a partially translocated form, since a significant portion of mAdDHFR was sensitive to the proteinase K-treatment (lanes 2 and 5; lanes 5 and 6). When the pAdDHFR-bound mitoplasts were solubilized by digitonin and subjected to immunoprecipitation, anti-Tim17 or Tim23 IgGs precipitated ~5% of the

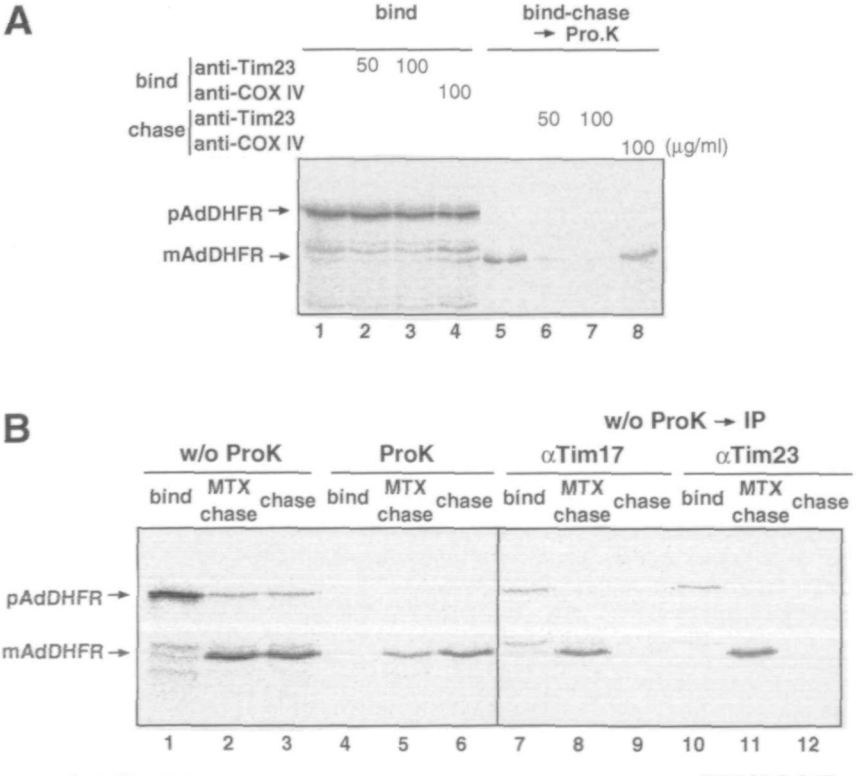

Fig. 4. **Effect of antibodies against Tim17 or Tim23 on binding or the subsequent import reactions of pAdDHFR into mitoplasts.** (A) Mitoplasts were incubated either without (lanes 1 and 5-8) or with (lanes 2-4) the indicated concentrations of antibodies against Tim23 or COX IV at 0°C for 30 min and subjected to the binding reaction of reticulocyte lysate-synthesized [^{35}S]-pAdDHFR at 10°C for 15 min ("bind"). The mitoplasts were reisolated by centrifugation and washed once with the homogenization buffer. The mitoplasts in lanes 5-8 were incubated with the indicated concentrations of IgGs against Tim23 or COX IV at 0°C for 30 min, then subjected to the import reaction at 30°C for 30 min. After import, the reaction mixtures were digested with proteinase K. The reaction mixtures were resolved by SDS-PAGE, and the gels were analyzed by FLA2000. (B) Mitoplasts were incubated with [^{35}S]-pAdDHFR at 10°C for 10 min, reisolated by centrifugation, and washed with the homogenization buffer (lanes 1-12). Where indicated the mitoplasts were then incubated with (lanes 2, 5, 8, and 11) or without (lanes 3, 6, 9, and 12) 10 μM methotrexate (MTX) at 30°C for 30 min ("chase"). The reaction mixtures in lanes 4-6 were then treated with proteinase K at 0°C for 30 min, whereas those in lanes 7-12 were dissolved by 0.25% digitonin, and the solubilized supernatants were subjected to immunoprecipitation with IgGs against Tim17 (lanes 7-9) or against Tim23 (lanes 10-12) as described in "MATERIALS AND METHODS." The reaction mixtures and the immunoprecipitates were resolved by SDS-PAGE and analyzed by FLA2000.

bound precursor and more than 20% of the processed form (lanes 7 and 10). When the chase reactions were performed in the presence of methotrexate (MTX), both anti-Tim17 and Tim23 IgGs efficiently precipitated the mature protein from digitonin-solubilized supernatant of the mitoplasts (30% of the translocation intermediates with anti-Tim23 IgGs) (lanes 8 and 11), whereas no precipitation was detected for the completely translocated mature protein that was produced after the chase reaction in the absence of methotrexate (lanes 9 and 12). These findings, along with the above results, indicate that most of the precursor bound to the mitoplasts at 10°C had not reached the Tim17-Tim23 step, and the presequence portion of most of the precursor had not crossed the inner membrane. The precursor is then transported into the mitoplasts through the Tim17-Tim23 system in the chase reaction, and mature AdDHFR associated with Tim17-Tim23 was only detected under the import-arrested condition with MTX.

We next measured the time course of the interaction of pAdDHFR with Tim23 after it had been targeted to the inner mitochondrial membrane by immunoprecipitation with anti-Tim23 IgGs. As shown in Fig. 5, the fraction of pAdDHFR associated with Tim23 increased over time and the reaction was also temperature-dependent. The binding of pAdDHFR to the mitoplasts also occurred with faster kinetics than the association of pAdDHFR with Tim23. Taken together, these results suggest that the precursor that was initially targeted to the inner membrane moved to the Tim23-Tim17 stage before translocation across the inner membrane.

A Synthetic Mitochondria-Targeting Signal Peptide Inhibits Both Precursor-Binding and Subsequent Chase Reactions—To probe the element in the precursor proteins that is recognized by the Tim translocation system, we examined the effect of a synthetic mitochondrial targeting signal peptide, SCC(1-19), on the precursor-binding and subsequent chase reactions. SCC(1-19) is a chemically synthesized functional mitochondrial signal peptide that specifically inhibits the binding of the precursors to MSF, Tom70, or Tom20 (*8*). As shown in Fig. 6A, SCC(1-19) inhibited both the precursor-binding and subsequent chase reactions in a dose-dependent manner. The trivial possibility that these inhibitions were brought about by dissipation of the membrane potential by SCC(1-19) could be ruled out, since 100 μM SCC(1-19), which is reported to partially dissipate the membrane potential (*38*), completely inhibited the precursor-binding and the chase reactions. Only a weak inhibition was observed with the control, nonfunc-

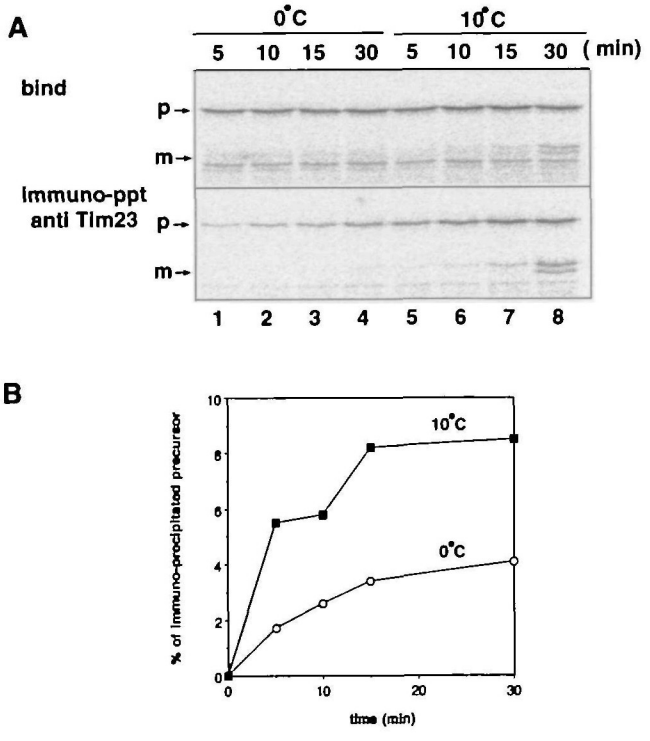

Fig. 5. **pAdDHFR forms a complex with Tim23 after being targeted to the inner mitochondrial membrane in a time- and temperature-dependent manner.** (A) Mitoplasts were incubated with reticulocyte lysate-synthesized [^{35}S]-pAdDHFR at 0°C (lanes 1-4) or 10°C (lanes 5-8) for the indicated times. The mitoplasts were reisolated by centrifugation ("bind"). The isolated mitoplasts were solubilized in 0.25% digitonin, and the supernatants were subjected to immunoprecipitation with antibodies against Tim23 ("immuno-ppt anti Tim23"). (B) The results in (A) were quantified with FLA2000, and the ratios of the co-precipitated pAdDHFR to mitoplast-bound pAdDHFR are shown.

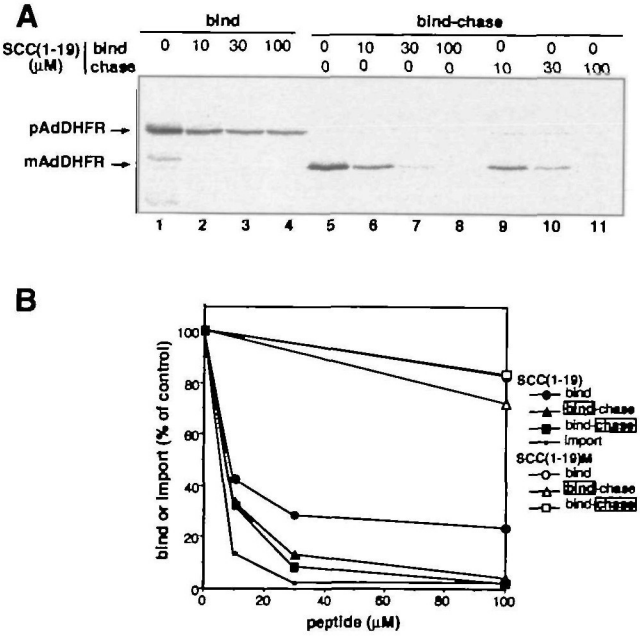

Fig. 6. **A synthetic mitochondria-targeting signal peptide, SCC(1-19), inhibits both the initial binding and the chase reactions into the mitoplasts.** (A) Reticulocyte lysate-synthesized [^{35}S]-pAdDHFR was incubated with mitoplasts in the presence (lanes 2-4 and 6-8) or absence (lanes 1, 5, and 9-11) of the indicated concentrations of SCC(1-19) at 10°C for 15 min. The mitoplasts (lanes 5-11) were reisolated and washed once with homogenization buffer. The mitoplasts in lanes 5-11 were subjected to the chase reaction at 30°C for 30 min in the absence (lanes 5-8) or presence (lanes 9-11) of the indicated concentrations of SCC(1-19), followed by proteinase K treatment. All the reaction mixtures were resolved by SDS-PAGE, and gels were analyzed by FLA2000. (B) Mitoplast-bound pAdDHFR and the imported form (mAdDHFR) were quantified by FLA2000. The efficiencies of the precursor-binding and the import were calculated by taking the signals of pAdDHFR in lane 1 and of mAdDHFR in lane 5 as 100%, respectively. The effects of control peptide SCC-(1-19)M on either the binding or subsequent import of pAdDHFR are also shown.

tional peptide SCC(1-19)M (*8*) (Fig. 6B). These observations indicate the presence of multiple MTS-recognizing steps in the process of precursor import into the mitoplasts. Approximately 20% of pAdDHFR recovered from the mitoplasts was resistant to 100 μM SCC(1-19) (Fig. 6B), whereas this fraction was not imported into the mitoplasts after the chase reaction (compare lanes 4 and 8), suggesting that the SCC(1-19)-resistant fraction may have been non-specifically adsorbed to the surface of the mitoplasts.

NaCl Inhibits the Precursor-Binding Reaction—We then examined the effect of salt concentrations on the precursor binding to the mitoplasts. As shown in Fig. 7, the overall import reaction of pAdDHFR was almost completely inhibited by 200 mM NaCl (Fig. 7A, lanes 9 through 12; Fig. 7B). On the other hand, ~30% of pAdDHFR remained bound to the mitoplasts at an NaCl concentration as high as 500 mM (Fig. 7), which probably reflects nonspecific binding to the inner membrane. Thus, the precursor binding to the mitoplasts is mainly mediated through ionic interactions. In marked contrast, the binding of AAC precursor (Fig. 7A, lanes 1 through 8) was increased by the addition of salt, and maximum import (lanes 9 through 12) was observed at 200 mM NaCl (Fig. 7). Nonionic interactions may contribute to the initial recognition of AAC precursor by the Tim22 system.

Both the Initial Binding of a Precursor and the Subsequent Import Require Membrane Potential across the Inner Membrane—We found that the initial binding and the

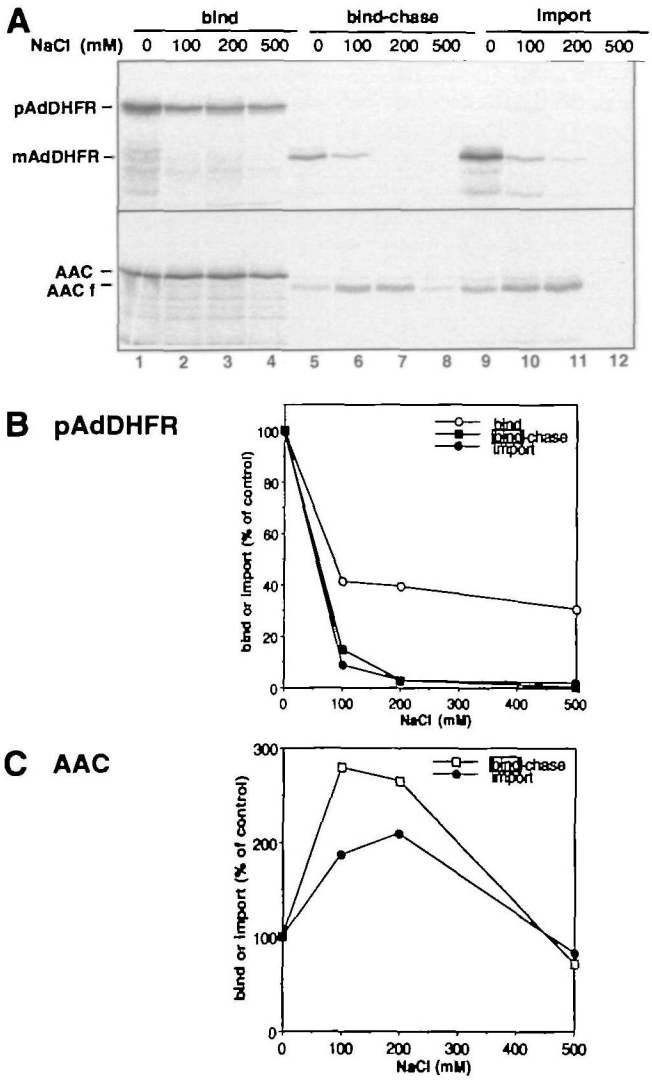

Fig. 7. **Binding to and import into mitoplasts of pAdDHFR are sensitive to salt-treatment, whereas those of AAC are insensitive to the treatment.** (A) *Reticulocyte lysate-synthesized* [^{35}S]-pAdDHFR or [^{35}S]-AAC was incubated with mitoplasts in the presence of the indicated concentrations of NaCl at 10°C for 15 min (lanes 1-8). The mitoplasts were isolated and washed with homogenization buffer. The mitoplasts in lanes 5-8 were subjected to the chase reaction at 30°C for 30 min, followed by proteinase K digestion. In a separate experiment, import of [^{35}S]-pAdDHFR or [^{35}S]-AAC into the mitoplasts was assayed in the presence of the indicated concentrations of NaCl, followed by proteinase K treatment (lanes 9-12). Other conditions were as described in "MATERIALS AND METHODS" and in Fig. 6. (B) The binding and import efficiencies of [^{35}S]-pAdDHFR were calculated by FLA2000. The efficiencies were calculated by taking the values in lanes 1, 5, and 9 as 100. (C) Binding and insertion efficiencies of [^{35}S]-AAC were calculated from (A) by FLA2000. All other conditions were same as those in (B).

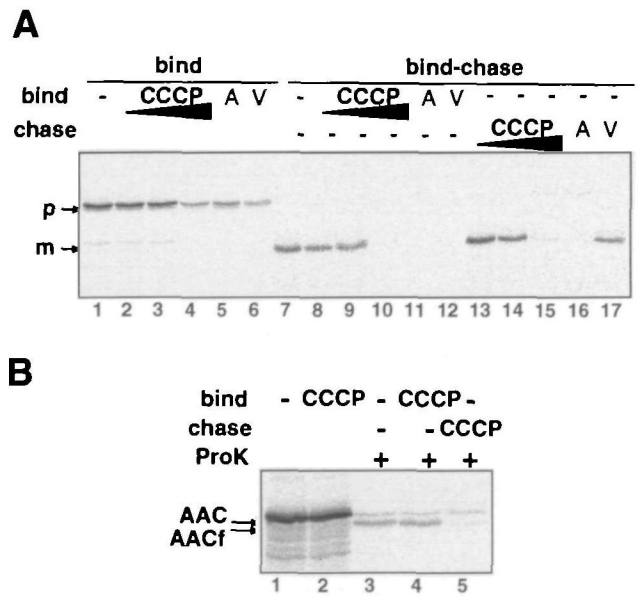

Fig. 8. **pAdDHFR requires $\Delta\Psi$ for binding to and translocation across the inner membrane, whereas AAC precursor requires $\Delta\Psi$ only in membrane integration.** (A) *Reticulocyte lysate*-synthesized [^{35}S]-pAdDHFR was incubated with the mitoplasts in the absence (lanes 1, 7, and 13-17) or presence of increasing concentrations of CCCP (1 nM, lanes 2 and 8; 100 nM, lanes 3 and 9; 10 μM, lanes 4 and 10), 10 μg/ml antimycin A (lanes 5 and 11), or 10 μg/ml valinomycin (lanes 6 and 12) at 10°C for 8 min. The mitoplasts were isolated by centrifugation and washed once with homogenization buffer. The mitoplasts in lanes 7-17 were then subjected to the chase reaction at 30°C for 30 min in the absence (lanes 7-12) or presence of CCCP (1 nM, 100 nM, and 10 μM for lanes 13, 14, and 15, respectively), 10 μg/ml antimycin A (lane 16), or 10 μg/ml valinomycin (lane 17). After import, the reaction mixtures were treated with proteinase K (lanes 7-17). (B) Reticulocyte lysate-synthesized [^{35}S]-AAC was incubated with the mitoplasts in the absence (lanes 1, 3, and 5) or presence (lanes 2 and 4) of 10 μM CCCP at 10°C for 15 min. The mitoplasts were isolated by centrifugation, washed once with homogenization buffer, then subjected to the chase reaction in the absence (lanes 3 and 4) or presence (lane 5) of 10 μM CCCP at 30°C for 30 min, followed by proteinase K digestion (lanes 3-5). All other conditions are the same as in Fig. 6.

subsequent chase reactions of pAdDHFR were both inhibited by CCCP (Fig. 8A). In this reaction, the time of precursor binding was set at 8 min to prevent the background production of mAdDHFR, since the background fraction had been imported into the matrix and lowered the signal *vs.* noise ratio. CCCP inhibited the binding of pAdDHFR to the mitoplasts dose-dependently (lanes 2-4). When these mitoplasts were reisolated, washed once with the homogenization buffer, and subjected to the import reaction, a dose-dependent inhibition of the import was observed (lanes 7-10), indicating that the productive binding of pAdDHFR was inhibited completely by CCCP. The CCCP-insensitive binding probably reflected the nonspecific adsorption of the precursor to the surface of mitoplasts (see also Figs. 6 and 7B). Furthermore, CCCP in the chase reaction inhibited the import of the mitoplast-bound pAdDHFR in a dose-dependent manner (lanes 13-15). These results indicate that both the initial binding of pAdDHFR and the subsequent import depended on the membrane potential across the inner membrane. The same results were obtained with valinomycin and antimycin, although valinomycin was, inexplicably, less effective in inhibiting the chase reaction of pAdDHFR (lanes 5, 6, 11, 12, 16, and 17). In contrast, the productive binding of AAC to the mitoplasts was not inhibited by CCCP, since the integration efficiency of AAC, as revealed by the production of the proteinase K-resistant fragment (AACf), was not affected by this treatment (Fig. 8B, lanes 1-4). On the other hand, the presence of CCCP during the chase reaction inhibited the insertion of AAC into the mitochondrial inner membrane (lane 5).

Effect of Anti-Tim23 IgGs, SCC(1-19), and CCCP on the Chase Reaction of the Mitoplast-Bound pAdDHFR—As described above, SCC(1-19) and CCCP inhibited both the precursor-binding and the chase reactions (Figs. 6 and 8), whereas anti-Tim23 inhibited only the chase reaction (Fig. 4A). We therefore investigated in more detail the inhibition steps by these reagents. For this purpose, we first prepared pAdDHFR-bound mitoplasts, which were subjected to the chase reaction in the presence of anti-Tim23 IgGs, SCC(1-19), or CCCP at 30°C for 30 min. The reaction mixtures were treated with or without proteinase K, then analyzed by SDS-PAGE and autoradiography. After the binding reaction at 10°C, pAdDHFR and a small amount of the intermediate processed form (an asterisk in the figure) were detected in the mitoplasts, both of which were sensitive to proteinase K (Fig. 9, lanes 1 and 2). After 30 min chase, pAdDHFR and the intermediate processed form were all imported into the matrix and processed to a mature form that was resistant to the externally added proteinase K (lanes 3 and 4). Anti-Tim23 IgGs did not inhibit the processing of the precursor (compare lanes 3 and 5).

However, the produced mAdDHFR was sensitive to externally added proteinase K (compare lanes 4 and 6), clearly indicating that anti-Tim23 did not inhibit the translocation of the presequence portion of the precursor but did inhibit the subsequent translocation of the mature portion of pAdDHFR. In contrast, both SCC(1-19) and CCCP inhibited the processing of the pre-bound pAdDHFR, indicating that they inhibited the translocation of the presequence portion across the inner membrane (lanes 7-10). It remains to be analyzed whether SCC(1-19) and CCCP inhibit the same step of the presequence translocation.

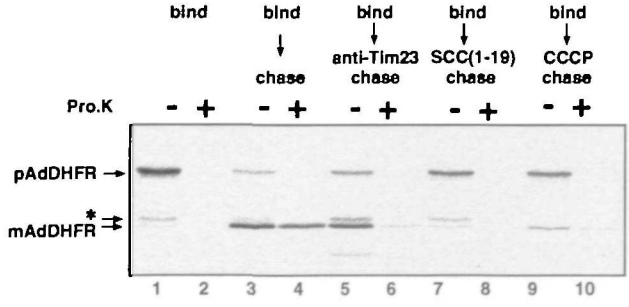

Fig. 9. **Effect of anti-Tim23 IgGs, SCC(1-19), or CCCP on the chase reaction of the mitoplasts-bound pAdDHFR.** Mitoplasts were incubated with reticulocyte lysate synthesized [^{35}S]-pAdDHFR at 10°C for 15 min, isolated by centrifugation, and washed once with homogenization buffer. The mitoplasts in lanes 3-10 were subjected to the chase reaction in the presence of 20 μg/ml anti-Tim23 IgGs (lanes 5 and 6), 30 μM SCC(1-19) (lanes 7 and 8), or 10 μM CCCP (lanes 9 and 10) at 30°C for 30 min. Where indicated, the reaction mixtures were treated with proteinase K (lanes 2, 4, 6, 8, and 10). The reaction mixtures were resolved by SDS-PAGE, and the gels were analyzed by FLA2000.

DISCUSSION

In comparison with our knowledge on yeast mitochondria, less is currently known about the mammalian mitochondrial protein import systems, particularly about the Tim system. Here, we have characterized the precursor import system of the rat liver mitochondrial inner membrane using mitoplasts. Rat liver mitoplasts exhibited a similar recognition specificity for MTS of matrix-targeted precursors to the Tom system, but exhibited faster kinetics in the precursor import.

Tim17 and Tim23 form a complex and are involved in the import of matrix-targeted precursors (*9-13, 21*), whereas Tim22 is involved in the import of metabolite carriers of the inner membrane in conjunction with Tim54, Tim10, and Tim12 (*17-20*). Since an EST database search revealed the presence of human and mouse homologues of Tim22, the precursor-translocation system of the inner membrane seems to be well conserved among species. In confirming these facts, the antibodies against rat Tim17 or rat Tim23 inhibited the import into mitoplasts of pAdDHFR but not AAC. Interestingly, SCC(1-19) did not inhibit the initial binding of AAC precursor to the mitoplasts, but it did inhibit the subsequent $\Delta\Psi$-dependent transport process (data not shown). The same inhibition was observed with the recombinant precursor: recombinant preadrenodoxin (pAd) inhibited $\Delta\Psi$-dependent integration of AAC into the mitoplasts, but recombinant porin, which is the major mitochondrial outer membrane protein, did not (data not shown). Thus, the components of the translocation machinery of rat mitoplasts (probably constituted from rat Tim22) for the metabolite-carrier precursors also recognize the matrix-targeting signal. These observations are consistent with the report in yeast that Tim23 carries an internal, positively charged import signal which potentially functions as the matrix-targeting signal. Nevertheless, Tim23 is inserted into the inner membrane *via* the Tim22 system, suggesting that both the Tim23-Tim17 and Tim22 systems

recognize similar import signals during the $\Delta\Psi$-dependent membrane-translocation step (*21*).

To analyze the Tim import system in more detail, we established a two-stage assay in which the overall import reaction was separated into the precursor-binding step at 0-10°C and the subsequent chase reaction at 30°C. This assay revealed four intermediate stages during precursor translocation across the inner membrane: stage I, precursor binds to the surface of mitoplasts (or loosely associates with Tim23-Tim17, stage IB); stage II, precursor associates with Tim23-Tim17; stage III, the presequence portion is translocated across the inner membrane, but most of the mature portion remains exposed to the exterior of mitoplasts; stage IV, mature protein is completely transported into the matrix (Fig. 10). This system enabled us to examine the individual effects of antibodies against Tim proteins, salt concentration, membrane potential, and synthetic mitochondria-targeting signal on both the binding and translocation of the precursor. It is reported in yeast that the intermembrane space domain of Tim23, which has an acidic leucine zipper motif, dimerizes in response to $\Delta\Psi$ and also functions as a precursor receptor (*13*). Unexpectedly, however, the antibodies against Tim23 inhibited neither the precursor binding nor the presequence translocation, but only the translocation of the mature segment of the precursor, even though they contained IgGs which recognize the intermembrane space domain of rat Tim23. The same results were obtained with selected IgGs that specifically recognized the intermembrane space domain of Tim23 (data not shown). In addition, the immunoprecipitation experiments revealed that the precursor at stage I did not interact (or interacted inefficiently) with Tim23-Tim17, but the precursor or mature protein at the later stages did. Interestingly, the initial stage of precursor binding depends on $\Delta\Psi$. The synthetic mitochondria-targeting signal peptide SCC(1-19) inhibited the formation of both stage I and stage III intermediates. Taken together, these observations suggest that the $\Delta\Psi$-dependent precursor receptor functions upstream of the Tim23-Tim17 stage. However, the present results do not clearly rule out the possibility that Tim23 not only acts as an initial binding site for preproteins on the surface of the inner membrane but also constitutes a part of the $\Delta\Psi$-dependent translocation channel (*13*); the initially targeted precursor may loosely associate with Tim23-Tim17, and Tim23-Tim17 may require conformational changes to achieve a stable association with the precursor (pathway B in Fig. 10).

What is the function of the $\Delta\Psi$-driven receptor of the inner membrane in mitochondrial protein import? The Tom and Tim machineries are believed to interact dynamically; they operate independently to transport the precursors to the outer membrane or the intermembrane space, or they couple to transport the precursors to the inner membrane or to the matrix space (*1-5*). In this context, the $\Delta\Psi$-driven receptor may function not only as a device to couple the Tom and Tim channels but as a precursor-sorting apparatus. Another possibility is that the $\Delta\Psi$-driven receptor may trigger opening of the Tim channel in conjunction with the presequence of incoming precursors, as suggested for yeast Tim23 (*13*). Future efforts must be directed to the characterization of the component which functions as the $\Delta\Psi$-driven initial receptor and the analysis of its function when the precursor is actively transported through the outer membrane into the inner membrane. It has been reported that acidic phospholipids such as cardiolipin partially unfold the precursor proteins and induce binding of the precursors to the mitochondria, while adriamycin, a drug binding to acidic phospholipids, specifically blocks this binding process (*39*). We examined this possibility and found that adriamycin (up to 300 μM) did not inhibit binding of pAdDHFR to the mitoplasts (data not shown), indicating that acidic phospholipids are not involved in this binding reaction, although the involvement of other phospholipids cannot be ruled out.

Most intriguingly, anti-Tim23 IgGs did not inhibit the $\Delta\Psi$-dependent translocation of the presequence across the inner membrane, but did inhibit the subsequent translocation step, in spite of the fact that both steps seemed to depend on the same channel activity of the Tim23-Tim17 system. What is the reason for this inhibition? One plausible explanation is that the conformation or oligomerization of the Tim23-Tim17 channel changes during the switch

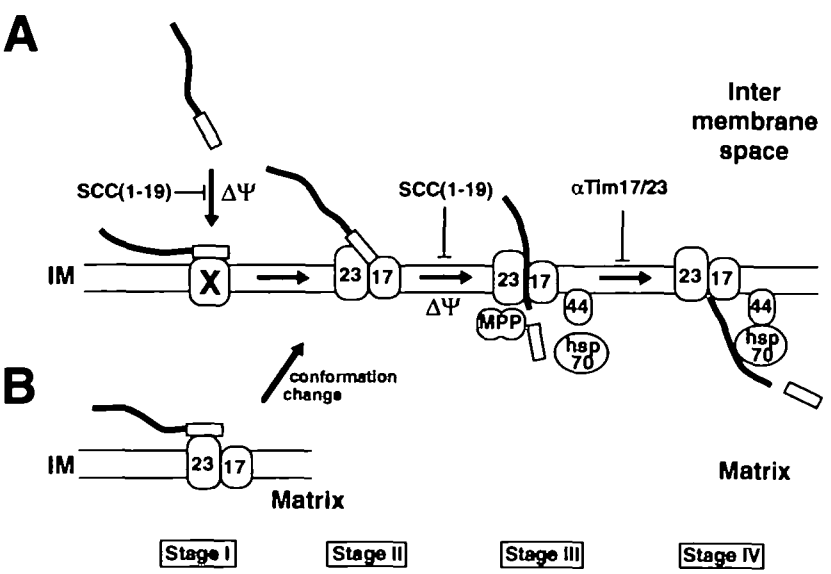

Fig. 10. A model for the steps of precursor translocation across the inner membrane of mammalian mitochondria. See text for details.

of the channel from the $\Delta\Psi$-coupled system to the mHsp70/Tim44-coupled system, and anti-Tim23 IgGs inhibit the latter state. It should be noted in this connection that the antibodies against the intermembrane space domain of yeast Tim23 prevented the import of pb2(169) DHFRk5 into yeast mitoplasts but failed to inhibit the chase reaction of MTX-arrested pb2(169)DHFRk5 (*12*). This discrepancy probably resulted from differences in the recognition specificity of the antibodies used.

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