The Import Route of ADP/ATP Carrier into Mitochondria Separates from the General Import Pathway of Cleavable Preproteins at the trans Side of the Outer Membrane*

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The ADP/ATP carrier (AAC) of the mitochondrial inner membrane is synthesized in the cytosol without a cleavable presequence. The preprotein preferentially binds to the mitochondrial surface receptor Tom70 and joins the import pathway of presequence-carrying preproteins at the cis side of the outer membrane. Little is known about the translocation of the AAC across the outer membrane and where its import route separates from that of cleavable preproteins. Here we have characterized a translocation intermediate of AAC during transfer across the outer membrane. The major portion of the preprotein is exposed to the intermembrane space, while a short segment is still accessible to externally added protease. This intermediate can be quantitatively chased to the fully imported form in the inner membrane. Its accumulation depends on Tom7, but not on the intermembrane space domain of Tom22 in contrast to cleavable preproteins. Moreover, opening of the intermembrane space inhibits the import of AAC, but not that of cleavable preproteins into mitoplasts. We conclude that the import route of AAC diverges from the general import pathway of cleavable preproteins already at the trans side of the outer membrane.

Most mitochondrial proteins are synthesized on cytosolic polysomes as preproteins with amino-terminal targeting sequences (presequences) that are cleaved off after import into the organelle (1–4). The ADP/ATP carrier (AAC)1 is the major representative of a large class of metabolite carriers of the mitochondrial inner membrane (5, 6) that are synthesized without presequences and contain targeting information within the mature protein parts (7, 8). Each carrier protein consists of about 300 amino acid residues, is predicted to contain six membrane-spanning segments, and is thought to have evolved by triplication of an ancient gene (5, 6, 9). The targeting signals in the carrier proteins are not exactly known. The available evidence indicates that the AAC contains at least two targeting signals distributed on distinct thirds of the preprotein (7, 8).

1 The ADP/ATP carrier (AAC) is also known as the ADP-ATP carrier, the ADP/ATP transporter, or the ADP/ATP translocase. It is a member of the family of mitochondrial carriers that transport metabolic intermediates across the mitochondrial inner membrane. The AAC is involved in the transport of ADP and ATP across the mitochondrial inner membrane, facilitating the electron transport chain and oxidative phosphorylation.

2 The progression of import of AAC through the mitochondrial import machinery involves multiple stages. Stage I involves the binding of AAC to the receptors Tom20 and Tom22 on the cis side of the outer membrane, leading to a transport intermediate that is protected against a treatment with trypsin (or low concentrations of proteinase K). Stage II involves the binding of AAC to the receptors on the cis side of the outer membrane (accumulation of AAC in the absence of Δψ and ATP); stage III involves the accumulation of AAC in mitochondria in the absence of a Δψ, but presence of ATP, leading to a transport intermediate that is protected against a treatment with trypsin. Stage IV involves the assembly of AAC in the inner membrane; stage V involves the translocation of AAC into the inner membrane.

3 The import route of AAC diverges from the general import pathway of cleavable preproteins already at the trans side of the outer membrane.
components have been identified and where mutant mitochondria are available. We describe a translocation intermediate of AAC during transfer across the outer membrane that can be quantitatively chased to the mature form in the inner membrane. The major portion of the translocation intermediate is exposed to the intermembrane space, while a small segment is still on the cytosolic side. Accumulation of the intermediate requires Tom7, but does not depend on the intermembrane space domain of Tom22. In contrast to cleavable preproteins, the productive accumulation of the translocation intermediate is insensitive to salt. Moreover, opening of the intermembrane space inhibits the import of AAC, but not that of cleavable preproteins. These results indicate that the import route of AAC diverges from the general import pathway already at the trans side of the outer membrane.

MATERIALS AND METHODS

The S. cerevisiae strains used are shown in Table I. Mitochondria were isolated as described by Daum et al. (25) and Hartl et al. (26). After in vitro transcription, preproteins were synthesized in rabbit reticulocyte lysates in the presence of [35S]methionine and [35S]cysteine. For the accumulation of the AAC at stage III, the mitochondria were incubated together with the radiolabeled preprotein in binding buffer (3% [w/v] BSA, 250 mM sucrose, 5 mM MgCl₂, 5 mM sodium malate, 20 mM KF, 1 μM valinomycin, 10 mM MOPS/KOH, pH 7.2) at 25 °C. (ATP was supplied from the reticulocyte lysate.) The mitochondria were either treated with proteinase K (150–200 μg/ml), reisolated, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (13) or were reisolated and incubated in chase buffer (3% [w/v] BSA, 250 mM sucrose, 5 mM MgCl₂, 5 mM sodium malate, 20 mM NaF, 1 μM valinomycin, 10 mM MOPS/NaOH, pH 7.2) at 25 °C to allow the AAC to be imported to its final location in the mitochondrial inner membrane. Mitochondria were then treated with proteinase K (27). For swelling, the mitochondria were reisolated and resuspended in EM buffer (10 mM MOPS/KOH, pH 7.2, 1 mM EDTA) or 10 mM Tris/HCl, pH 7.4, for 15 min at 4 °C (in case of a subsequent proteinase K treatment, 50 μg/ml were used). In cases where preproteins were directly imported without an accumulation at the outer membrane, import was performed in BSA buffer (3% [w/v] BSA, 250 mM sucrose, 5 mM MgCl₂, 80 mM KF, 10 mM MOPS/KOH, pH 7.2) containing 2 mM ATP and 2 mM NADH. To dissipate the membrane potential, a mixture of 1 μM valinomycin, 20 μM oligomycin, and 8 μM antimycin A was added to the import reaction.

For the immunoprecipitation of the AAC, antibodies were added either directly to the mitochondria after the import reaction or to lysed mitochondria (28). Mitochondria were lysed with Triton X-100 buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.5, 300 mM NaCl). The lysed material was subjected to a clarifying spin, applied onto protein A-Sepharose, and incubated for 1 h. The bound material was eluted and analyzed by SDS-PAGE.

Alkaline extraction of mitochondria (100 mM Na₂CO₃) and separation of pellet and supernatant were performed as published (27). For the salt extraction of accumulated AAC, mitochondria were incubated with radiolabeled AAC in binding buffer for 15 min at 25 °C. Samples were split into two aliquots and were either reisolated or first treated with proteinase K and then reisolated. The pelletted mitochondria were resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) containing different concentrations of NaCl and incubated for 15 min at 4 °C. For sonication, the mitochondria were resuspended in SEM buffer with 200 mM NaCl and sonified for 6 × 6 s in a Branson sonifier 250 (50% duty cycle) (29), followed by separation into pellet and supernatant by centrifugation at 266,000 × g for 90 min at 2 °C and precipitation with 7.5% trichloroacetic acid in the presence of 0.0125% sodium deoxycholate.

Standard procedures were used for Western blotting onto nitrocellulose using enhanced chemiluminescence system (Amersham Pharmacia Biotech) and storage phosphorimaging technology (Molecular Dynamics).

RESULTS

Accumulation of AAC at the Intermembrane Space Side of the Outer Membrane—The precursor of AAC was synthesized in rabbit reticulocyte lysates in the presence of [35S]methionine/cysteine and incubated with isolated yeast mitochondria in the presence of ATP, but the absence of a Δψ across the inner membrane. The AAC accumulated at this stage III is protected against trypsin (or a low concentration of proteinase K) added to the mitochondria (13, 30, 31) but becomes accessible to the protease after opening of the intermembrane space by swelling of mitochondria and rupturing of the outer membrane (formation of mitoplasts) (30). This result suggested that the preprotein may be exposed to the intermembrane space. It could not be excluded, however, that the preprotein was enclosed by proteinclose components of the import machinery and became accessible to the intermembrane space side only after proteolytic degradation of protecting components. We therefore applied a non-destructive assay to test for the localization of AAC, that is the accessibility to antibodies (we previously showed that AAC accumulated at the surface receptors (cis side of the outer membrane; no treatment with protease) was, as expected, accessible to antibodies (28)).

Deenergized mitochondria with AAC accumulated at stage III were treated with proteinase K at a low concentration (10 μg/ml) to remove surface-bound preproteins. The mitochondria were reisolated, and the intermembrane space was opened. Then antibodies specifically directed against the AAC were added. After an incubation, antibodies that were not bound to the mitochondria were removed by reisolation of the mitochondria. Then the mitochondria were lysed in Triton X-100-containing buffer, and immunocomplexes were harvested by protein A-Sepharose. By this procedure we found that the accumulated AAC was efficiently recognized by the antibodies upon opening of the intermembrane space (Fig. 1A, upper panel, lane 2), while it was not accessible to the antibodies in intact mitochondria (Fig. 1A, upper panel, lane 1). The lack of immunoprecipitation from intact mitochondria excludes that antibodies added prior to lysis and reisolation of mitochondria became active in immunoprecipitation after the lysis. The total amount of immunoprecipitable AAC was then determined by addition of antibodies after the lysis of the mitochondria (Fig. 1A, middle panel, lanes 1–4). AAC completely imported into the inner membrane in the presence of a Δψ was not accessible to antibodies even after swelling (Fig. 1A, upper panel, lanes 3 and 4); it was precipitated only by antibodies added after lysis (Fig. 1A, middle panel, lanes 3 and 4). We conclude that AAC accumulated at stage III is exposed to the intermembrane

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**TABLE I**

| Strain         | Genotype                                   | Reference |
|---------------|--------------------------------------------|-----------|
| YPH499 (wild-type) | ade2–101 his3–Δ200 leu2–Δ1 ura3–52 trp1–Δ63 lys2–801 | 47        |
| MM307 (tom6Δ)  | ade2–101 his3–Δ200 leu2–Δ1 ura3–52 trp1–Δ63 lys2–801 tom6::URA3 | 37        |
| AH101 (tom7Δ)  | ade2–101 his3–Δ200 leu2–Δ1 ura3–52 trp1–Δ63 lys2–801 tom7::TRP1 | 27        |
| AH610 (tom6Δ tom7Δ) | ade2–101 his3–Δ200 leu2–Δ1 ura3–52 trp1–Δ63 lys2–801 tom6::URA3 tom7::TRP1 | 27        |
| OL200-AH49 (tom22–2) | his3–Δ200 leu2–Δ1 ura3–52 trp1–Δ63 tom22::HIS3 + pRS414/TRP1::tom22–2 | 39        |
Import of ADP/ATP Carrier into Mitochondria

Fig. 1. The ADP/ATP carrier (AAC) accumulated in deenergized mitochondria (stage III) is associated with the outer membrane and exposed to the intermembrane space. A, accessibility of AAC to antibodies. Reticulocyte lysate with 35S-labeled AAC was incubated with isolated S. cerevisiae mitochondria in the presence or absence of a membrane potential \( \Delta \psi \) for 10 min. The samples were treated with 10 \( \mu \)g/ml proteinase K. The reaction was stopped by the addition of 1 m\( \text{M} \) phenylmethylsulfonyl fluoride. The samples were split. One aliquot was directly incubated with antiserum directed against AAC, in the other aliquot the mitochondria were first swollen and then incubated with antibodies. After reisolation, mitochondria were lysed in Triton X-100-containing buffer. After a clarifying spin, protein A-Sepharose was added, and antibody-AAC complexes were harvested and analyzed by SDS-PAGE and autoradiography (upper panel). The supernatant (after removal of the protein A-Sepharose by centrifugation) was added to new protein A-Sepharose that had been preincubated with anti-AAC serum, representing the material where antibody molecules can efficiently bind to AAC at stage III is membrane-integrated (Fig. 1, upper panel, lane 1) or fully imported AAC (stage V; mitoplasts) (Fig. 1, lower panel, lane 1). Since we will show below that the AAC molecules giving rise to these fragments can be quantitatively chased to the fully imported form, they represent true translocation intermediates at stage III and are henceforth referred to as AACIII. The proteinase K treatment degraded surface-exposed components of the protein import machinery, such as Tom20 (Fig. 1, upper panel, lane 6), but did not affect the outer membrane barrier, as evidenced by the full protection of the intermembrane space protein cytochrome b\( _{2} \) (Fig. 1, lower panel, lane 6) and of AAC transported into the inner membrane (Fig. 1, lower panel, lane 2) (31). After opening of the intermembrane space by swelling, AACIII was no longer protected against the protease but completely degraded (Fig. 1, lower panel, lane 3). We did not observe fragments that could be resolved by the SDS-PAGE suggesting that AAC at stage III is exposed to the intermembrane space with major portions.

In parallel reactions, we imported the precursor of AAC into energized mitochondria (stage V) (Fig. 1, lower panel, lane 2). A treatment with proteinase K did not lead to generation of AACIII (Fig. 1, lower panel, lane 2). After swelling of the mitochondria, however, the proteinase K cleaved off a small piece (\( \sim 1 \) kDa) from the imported AAC (Fig. 1, lower panel, lane 4). This fragmentation in swollen mitochondria is indicative of fully imported AAC (21, 24, 30) and is thus referred to as AACV.

We conclude that AAC at stage III is mainly exposed to the intermembrane space but remains in stable contact with the outer membrane and exposes a small segment to the cytosolic side.

AAC Accumulated at Stage III Is Quantitatively Chased to the Mature Form—We asked if AAC giving rise to AACIII represented a true translocation intermediate, i.e. if the accumulated AAC could be chased to the fully imported form after regeneration of \( \Delta \psi \). The 35S-labeled precursor of AAC was accumulated in deenergized mitochondria (dissipation of \( \Delta \psi \) by valinomycin in the presence of potassium in the incubation buffer) with three different incubation times. In a first set, the accumulated AAC was directly analyzed (Fig. 2, lanes 1-6); a treatment of the mitochondria with proteinase K generated AACIII (Fig. 2, lanes 1-3), whereas after swelling proteinase K space such that entire antibody molecules can efficiently bind to it.

To assess the localization of AAC accumulated in deenergized mitochondria, two assays were applied, treatment at alkaline pH and sonication. A treatment of the mitochondria at pH 11.5 (sodium carbonate) releases soluble proteins and peripheral membrane proteins (bound by protein-protein interactions) to the supernatant and leaves integral membrane proteins (protein-lipid interaction) in the membrane sheets (32, 33). This treatment released the AAC (stage III) to the supernatant (Fig. 1B, column 2), while AAC completely imported into the inner membrane in the presence of a \( \Delta \psi \) (stage V) remained membrane-integrated (Fig. 1B, column 3) (11, 27). To test if the AAC accumulated at stage III was soluble in the intermembrane space or still membrane-bound, the mitochondria were sonicated (29), and soluble proteins and membrane vesicles were separated. The AAC was found to be stably associated with the membrane vesicles (Fig. 1B, column 1). Together with the release at pH 11.5, this result suggests that the AAC at stage III is bound to the membranes by protein-protein interactions.

Is the AAC intermediate still in contact with the mitochondrial outer membrane? Deenergized mitochondria with accumulated AAC (Fig. 1C, upper panel, lane 1) were treated with proteinase K (150 \( \mu \)g/ml), leading to the generation of fragments of AAC that were 2–4 kDa smaller than the full-length protein (Fig. 1C, lower panel, lane 1). Since we will show below that the AAC molecules giving rise to these fragments can be quantitatively chased to the fully imported form, they represent true translocation intermediates at stage III and are henceforth referred to as AACIII. The proteinase K treatment degraded surface-exposed components of the protein import machinery, such as Tom20 (Fig. 1C, upper panel, lane 6), but did not affect the outer membrane barrier, as evidenced by the full protection of the intermembrane space protein cytochrome b\( _{2} \) (Fig. 1C, lower panel, lane 6) and of AAC transported into the inner membrane (Fig. 1C, lower panel, lane 2) (31). After opening of the intermembrane space by swelling, AACIII was no longer protected against the protease but completely degraded (Fig. 1C, lower panel, lane 3). We did not observe fragments that could be resolved by the SDS-PAGE suggesting that AAC at stage III is exposed to the intermembrane space with major portions.

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completely degraded AACIII; no fully imported AAC was detected (Fig. 2, lanes 4-6). In a second set (Fig. 2, lanes 7-12), the mitochondria with accumulated AAC were reisolated and subjected to a second incubation in the presence of a Δψ by generating a potassium diffusion potential (valinomycin in the absence of potassium in the buffer (27, 34, 35)). By this “chase incubation,” the AACIII completely disappeared (non-swollen mitochondria; Fig. 2, lanes 7-9). After swelling of the mitochondria, proteinase K did not degrade AAC, but led to removal of a small fragment in a considerable fraction of the AAC (Fig. 2, lanes 10-12), i.e. AACIV was generated. This demonstrates that AAC accumulated at stage III (AACIII) was efficiently chased to the fully imported form.

Does the chase reaction involve the function of surface-exposed (cis) components of the protein import machinery of the outer membrane? We took advantage of the observation that AAC accumulated at stage III is resistant to a treatment of mitochondria with trypsin under conditions that lead to a removal of the cytosolic domains of the surface receptors Tom20, Tom22, and Tom70 (13, 30, 31). Therefore, in a third set of samples, the AAC was accumulated in energized mitochondria, followed by a trypsin treatment (Fig. 2, lanes 13-18). Then the mitochondria were reisolated and subjected to the chase incubation (generation of a Δψ). Lanes 13-18 of Fig. 2 demonstrate that the AAC accumulated at stage III was efficiently chased to the fully imported form (AACV), also after removal of the cytosolic domains of Tom20, Tom22, and Tom70.

Accumulation of AACIII Requires Tom7, but Not the Intermembrane Space Domain of Tom22—We asked if other components of the Tom machinery were involved in the accumulation of AACIII. Two small Tom proteins have been identified that modulate the dynamics of the outer membrane translocase. Studies with deletion mutants of yeast showed that Tom6 (36) promotes association of components of the Tom machinery (37) whereas Tom7 supports a dissociation of the Tom machinery (27). tom6Δ mitochondria were able to generate AACIII (Fig. 3A, upper panel, lane 1) that was degraded by proteinase K after swelling of the mitochondria (Fig. 3A, lower panel, lane 1). In contrast, with tom7Δ mitochondria no AACIII was observed (Fig. 3A, upper panel, lane 2). The tom7Δ mitochondria were not unspecifically damaged since they were still able to import AAC in the presence of a Δψ, including formation of the fragment AACV (Fig. 3A, lower panel, lane 6), although with a reduced efficiency (27). Moreover, proteinase K formed fragments of AAC in swollen mitochondria after import in the presence of a Δψ that were 5–7 kDa smaller than full-length AAC (AACIV, Fig. 3A, lower panel, lane 6). These fragments, indicating incomplete insertion of AAC into the inner membrane, were found in only small amounts in wild-type and tom6Δ mitochondria (Fig. 3A, lower panel, lane 5; Fig. 3B, columns 1 and 2), but were significantly more abundant in tom7Δ mitochondria (Fig. 3A, lower panel, lane 6; Fig. 3B, column 3). This result supports an impairment of tom7Δ mitochondria in import of AAC.

Tom6 and Tom7 function in an antagonistic manner in modulating the dynamics of the Tom machinery (27). If the inhibitory effect of a lack of Tom7 on the generation of AACIII was due to the role of Tom7 in dissociation of the Tom machinery, mitochondria from a double deletion strain tom6Δ tom7Δ (27) should be able to generate some AACIII intermediate. Lane 3 of Fig. 3A (upper panel) shows that this was indeed the case. We conclude that Tom7 is required for generation of AACIII in a manner antagonistic to the function of Tom6. Moreover, in the presence of a Δψ, the formation of the AACIV fragments, indicative of incomplete insertion of AAC into the inner membrane, was reduced in tom6Δ tom7Δ mitochondria (Fig. 3A, lower panel, lane 7; Fig. 3B, column 4) compared with tom7Δ mitochondria (Fig. 3, lower panel, lane 6; Fig. 3B, column 3).

The intermembrane space domain of Tom22 is critical for accumulation of presequence-containing preproteins at a trans site of the outer membrane, as shown with mutant mitochondria (tom22-2) selectively lacking this domain (38, 39). We asked if the formation of AACIII required the same component at the intermembrane space side. tom22-2 mitochondria were not impaired in generation of AACIII (Fig. 3, upper panel, lane 4), suggesting that the trans sites for presequence-containing preproteins and AAC are different.

To obtain independent evidence for a difference in trans site accumulation, we assayed for a further characteristic of import of presequence-containing preproteins. The productive accumulation of a cleavable preprotein at the trans site of the outer membrane, determined by accumulation in the absence of a Δψ and subsequent chase to the imported form (two-step import), is only partially resistant to a treatment of mitochondria with salt, i.e. resistant to 100 mM NaCl, but inhibited at higher salt concentration; at 200 mM NaCl, the productive accumulation of a cleavable preprotein is blocked (39). We showed above that AAC accumulated at stage III remains membrane-associated during sonication at 200 mM NaCl, suggesting a difference
to cleavable preproteins. To directly determine if the salt-resistant AAC represented a productive intermediate, we assayed for the two-step import of AAC at distinct salt concentrations. Mitochondria with AAC accumulated in the absence of a Δψ for 15 min at 25 °C. After reisolation of the mitochondria, the samples were incubated in binding buffer described under “Materials and Methods” supplemented with different concentrations of sodium chloride as indicated for 15 min at 4 °C. Each sample was split into three aliquots, respectively, of which the third contained double the volume. One aliquot was directly treated with proteinase K, and the mitochondria were reisolated and analyzed by SDS-PAGE and digital autoradiography. In the second aliquot, the mitochondria were swollen (see “Materials and Methods”), and the resulting mitoplasts were treated with proteinase K. The third aliquot was incubated in chase buffer (presence of a Δψ) for 20 min at 25 °C and was then split into two halves. One half was directly treated with proteinase K; in the other half, the mitochondria were first swollen and then treated with the protease.

Opening of the Intermembrane Space Inhibits the Import of AAC, but Not of a Cleavable Preprotein—After swelling of mitochondria, cleavable preproteins have been shown to be directly translocated across the inner membrane without a requirement for the Tom machinery of the outer membrane (40, 41). We compared the import of the precursor of F1-ATPase subunit β (F1β) to that of AAC into mitoplasts. To prevent the use of the Tom machinery, mitochondria were pretreated with trypsin to remove the surface receptors. Thereby the import of both F1β and AAC into non-swollen mitochondria was strongly inhibited (Fig. 5, lower panel, lanes 2 and 8; compare with lanes 1 and 7 (no trypsin treatment)). After swelling of the trypsin-treated mitochondria, the import of F1β was restored to a large extent, demonstrating direct translocation across the inner membrane in a Δψ-dependent manner (Fig. 5, lanes 3). In contrast, the import of AAC into the mitochondria was still strongly inhibited (Fig. 5, lower panel, lane 9).

Two possibilities are conceivable to explain this import inhibition of AAC, the lack of interaction with the outer membrane machinery or a loss of intermembrane space function required for import of AAC. To distinguish between the possibilities, non-trypsinized mitochondria were subjected to swelling and subsequently incubated with preproteins. The import of F1β was very efficient (Fig. 5, lanes 5), whereas the import of AAC was still inhibited (Fig. 5, lower panel, lane 11). Under these conditions, the AAC can use the intact Tom machinery, but is still unable to translocate into the inner membrane. We con-
clude that opening of the outer membrane by swelling leads to a loss of an intermembrane space function that is essential for import of AAC.

DISCUSSION

We have identified a translocation intermediate of the AAC during transfer of the preprotein across the yeast mitochondrial outer membrane and passage into the intermembrane space. Thereby the first detailed characterization of the import stage III of a mitochondrial metabolite carrier, i.e. the transport pathway between the recognition of the preprotein at the outer membrane surface (stage II) and the insertion into the inner membrane (stage IV), has been possible (Fig. 6). Previous work has indicated that stage III intermediates, accumulated in the absence of a Δψ, are protected against trypsin added to the isolated mitochondria (11–13, 31) but become accessible to the protease after opening of the intermembrane space (30). However, most characteristics of the stage III intermediates have been unknown, such as the topology, the dependence on Tom components, and the relation to the import pathway of presequence-containing preproteins.

The stage III intermediate of AAC is stably membrane-bound, although not as integral membrane protein, but apparently by protein-protein interactions. The preprotein exposes major parts to the intermembrane space such that entire antibody molecules have access to the preprotein after swelling of mitochondria. A striking observation is the cleavage of the stage III intermediate by proteinase K (150 μg/ml added to non-swollen mitochondria) to a few fragments of closely related length, termed AACIII (2–4 kDa smaller than full-length AAC). This demonstrates that the stage III intermediate spans the outer membrane and exposes a short segment to the cytosolic side. In a chase reaction after establishing a Δψ, all AAC molecules that can give rise to the formation of AACIII are quantitatively chased to the fully imported form. This remarkably high chase efficiency demonstrates that AACIII is indicative of a true translocation intermediate. For the accumulation of the stage III intermediate, the Δψ across the inner membrane is selectively dissipated while the other known requirements for import, in particular the presence of ATP, are not impaired. The formation of the AACIII fragments shows that the stage III intermediate is still spanning the outer membrane, demonstrating that the prevention of AAC insertion into the inner membrane inhibits the complete translocation across the outer membrane. This suggests a coupling between the completion of outer membrane translocation and the Δψ-dependent insertion into the inner membrane.

Tom7, a small subunit of the outer membrane translocase, has been shown to support a dissociation of the Tom machinery (27). We find here that Tom7 is important for the generation of AACIII. Tom6 functions in an antagonistic manner to Tom7, in that Tom6 promotes an association of components of the Tom
biogenesis. It was found very recently that both proteins are required for import of carrier proteins, but not of cleavable preproteins (45, 46). A loss of these intermediate space preproteins, in particular Tim10, by swelling of mitochondria can explain the striking difference of import of cleavable preproteins and AAC into mitoplasts.

The implication derived from characterization of AACIII that the import pathways of cleavable preproteins and carrier proteins diverge early is thus supported by the differential requirement for intermediate space components. We conclude that AAC and cleavable preproteins use common components for insertion into the general import pore of the outer membrane, but that already at the exit of the general import pore the pathways separate and probably remain distinct for transfer through the intermediate space and insertion into the inner membrane.

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