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The Dynamic Dimerization of the Yeast ADP/ATP Carrier in the Inner Mitochondrial Membrane Is Affected by Conserved Cysteine Residues*

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The ADP/ATP carrier (AAC) that facilitates the translocation of ATP made in mitochondria is inserted at the inner mitochondrial membrane by the TIM10-TIM22 protein import system. Here we addressed the state of the AAC precursor during insertion (stage IV of import) and identified residues of the carrier important for dimerization. By a combination of (i) import of a mix of His-tagged and untagged versions of AAC either ^{35}S-labeled or unlabeled, (ii) import of a tandem covalent dimer AAC into wild-type mitochondria, and (iii) import of monomeric AAC into mitochondria expressing only the tandem covalent dimer AAC, we found that the stage IV intermediate is a monomer, and this stage is probably the rate-limiting step of insertion in the membrane. Subsequent dimerization occurs extremely rapidly (within less than a minute). The incoming monomer dimerizes with monomeric endogenous AAC suggesting that the AAC dimer is very dynamic. Conserved Cys residues were found not to affect insertion significantly, but they are crucial for the dimerization process to obtain a functional carrier.

The ADP/ATP carrier (AAC)[1] is one of the most abundant proteins present in the inner mitochondrial membrane (1). It catalyzes the transmembrane exchange of cytosolic ADP^{3-} for mitochondrial ATP^{4-}, the latter generated by oxidative phosphorylation. This protein is composed of three homologous modules, where each module consists of two membrane-spanning domains connected by a large hydrophilic loop extending into the matrix. AAC is a member of the mitochondrial carrier family that contains at least 40 different polypeptides in yeast with distinct substrate specificities (2).

Unlike the majority of proteins imported into mitochondria that contain N-terminal presequences, AAC is synthesized without a cleavable extension in most species (3–5). Instead, the three structurally related modules of this carrier contain internal targeting signals that facilitate import and insertion into mitochondria (6, 7).

The import of inner membrane metabolite carriers such as the AAC can be divided into five intermediate stages (8, 9). The synthesis of the preprotein in the cytosol, complexed with molecular chaperones is referred to as stage I. Stage II involves the binding of individual AAC modules to Tom 70, which is proposed to function as a specific receptor for carriers (10, 11). The translocation of AAC through the general import pore of the outer mitochondrial membrane (stage IIIa) may be facilitated by the formation of loop structures (7, 12). Loop formation may enable interaction between receptors and internal targeting signals, which would be physically separated if AAC imported as a linear polypeptide. The TIM10 complex, composed of Tim9 and Tim10 proteins subsequently binds to AAC, mediating the passage of the hydrophobic carrier across the intermembrane space (13–18). In doing so, the TIM10 complex presumably prevents aggregation in the aqueous intermembrane space in agreement with a recently demonstrated chaperone-like function *in vitro* (19). This carrier-chaperone complex is then guided to Tim12, which is peripherally attached to the outer surface of the inner membrane (stage IIIb). Tim12 is part of the membrane-embedded TIM22 complex, which is composed of the three integral membrane proteins, Tim22, Tim54, and Tim18 (20–23). Insertion of AAC into the TIM22 channel requires a membrane potential (stage IV), and its assembly into a functional homodimer in the membrane is referred to as stage V. Although Tim22 has been shown to be the key component for insertion of AAC (24), the nature of the stage (IV) intermediate and the mechanism of dimerization have not yet been elucidated.

The translocation function of the ADP/ATP carrier is dependent on the formation of a 12 transmembrane assembly, which, in the case of yeast AAC, is ensured by the formation of a dimer between two six transmembrane monomers. An alternative arrangement is exemplified by the adenylate nucleotide translocator of the obligate parasite *Rickettsia prowazekii* and plastids of *Arabidopsis thaliana* that exhibit a single polypeptide of 12 putative transmembrane domains (25). The presence of a

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[1] The abbreviations used are: AAC, ADP/ATP carrier; PMSF, phenylmethylsulfonyl fluoride; NTA, nitrilotriacetic acid; DTT, dithiothreitol.

twelve transmembrane assembly to mediate small molecule translocation across a membrane is quite common among other translocation systems (26–30) and it is a thermodynamically favored arrangement (31).

Previous work using purified metabolite carriers (32) had suggested that a cross-linked dimer has the capacity to insert into an artificial lipid bilayer. However, it is unknown whether *in vitro* synthesized, nascent AAC inserts as a monomer and subsequently assembles into a functional homodimer, or whether it inserts in a "pre-assembled" dimeric form in the mitochondrion. Additionally, the presence of conserved cysteine residues in yeast AAC indicates that dimeric formation may be dependent on intermolecular disulfide bonds, and/or facilitated by other Cys-specific chemical interactions. These are important questions that underpin the recognition of AAC by the specific TIM translocases during the different stages of import *in vivo*.

In the present study we report that the precursor of the stage IV intermediate is a monomer. Subsequently, we demonstrate that the incoming monomeric precursor forms a dimer with preinserted endogenous AAC very rapidly (less than a minute under our experimental conditions), hence suggesting that the endogenous dimer exists in a dynamic form. The dimeric, mature form of this protein is resistant to reducing agents indicating intermolecular disulfide bonds are absent from the final structure, but the dimerization process is affected in cysteine mutants. This implicates the importance of cysteine residues in the proper assembly of dimeric AAC.

EXPERIMENTAL PROCEDURES

Construction of the His-tagged AAC1 Plasmid—To introduce an N-terminal His tag to AAC1, the open reading frame was amplified from the plasmid pSP64-AAC1 with Deep Vent polymerase (New England Biolabs) using the polymerase chain reaction with the primers AACF (CGC CAT ATG CAT CAC CAT CAC CAT CAC TCT CAC ACA GAA ACA CAG) and AACR (CGC GAA TTC TCA CTT GAA TTT TTT GCC AAA CAT TAT GAG TTG). The PCR product was ethanol-precipitated, digested with the restriction enzymes *Nde*I and *Eco*RI, gel-purified, and ligated into the pET21b vector (Novagen) previously cut with *Nde*I and *Eco*RI to create the clone pET AAC2.2.

Construction of Cysteine Mutants of AAC1—All mutants were made using the QuickChange mutagenesis kit (Stratagene) to introduce single base mutations to change cysteine to serine codons, with the pSP64-AAC1 plasmid as template. For mutating the cysteine residue at position 63 (CS63), and creating the clone pSP64-M2.1, the primers CS63+ (ACA AGG GAA TTT TAG ATT CCT TCA AGA GGA CTG CGA C) and CS63− (GTC GCA GTC CTC TTG AAG GAA TCT AAA ATT CCC TTG T) were used. For mutating the cysteine residue at position 262 (CS262), and creating the clone pSP64-C5.4, the primers CS262+ (ACG ACG GTG CTC TGG ACT CTT TGA GAA AGA TTG TTC A) and CS262− (TGA ACA ATC TTT CTC AAA GAG TCC AGA GCA CCG TCG T) were used. For mutating the cysteine residue at position 279 (CS279), and creating the clone pSP64-C2.1, the primers CS279+ (ATT CCT TGT TCA AGG GCT CTG GTG CCA ACA TAT TTA G) and CS279− (CTA AAT ATG TTG GCA CCA GAG CCC TTG AAC AAG GAA T) were used. Underlined nucleotides show the position of the mutation. To generate the triple cysteine mutant plasmid pSP64-muC3 (CS3X), two rounds of mutation were performed on the plasmid pSP64-M2.1 (CS63) using the primer pairs CS262+/CS262− and CS279+/CS279−.

Construction of AAC2 Plasmids—Plasmid $(AAC2p)_2$ was constructed by insertion of a 2.4-kb *EcoR1-Sal* fragment of KSDIM5′3′ (35) containing two repeats of the *ANC2* open reading frame into pSP65 (Promega), digested with the same restriction enzymes.

Plasmid AAC2p was constructed by amplifying yeast genomic DNA using the oligonucleotides, AAC2p+ (CCG GAA TTC ATG TCT TCC AAC GCC CAA GCT) and AAC2p− (GCG GGA TCC TTA TTT GAA CTT CTT ACC AAA C). Following *EcoR1-BamH1* digestion, the PCR product was subcloned into pSP65, digested with the same restriction enzymes.

In Vitro Synthesis of Precursor Radiolabeled Proteins and Import into Isolated Mitochondria—Mitochondria were purified from lactate-grown wild-type (D273-10B) or $(JL1\text{-}3\text{-}(AAC2)_2$ *Saccharomyces cerevisiae* cells as described in Refs. 33 and 34, respectively. Proteins were synthesized *in vitro* from plasmid using a coupled transcription/translation system with rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. Protein was synthesized from the pET AAC2.2 clone using T7 RNA polymerase (Amersham Biosciences). For synthesis of cold protein, the [^{35}S]methionine was replaced by 1 mM unlabeled methionine. For synthesis from plasmids pSP64-AAC1, pSP64-M2.1, pSP64-C5.4, pSP64-C2.1 and pSP64-muC3, SP6 RNA polymerase was used.

Mitochondria were preincubated for 5 min at 25 or 30 °C prior to import of the precursor. Lysate containing the precursor at a final 5–10% concentration was incubated at 25 or 30 °C for 15 min with 0.1 mg of purified mitochondria in import buffer containing 0.6 M sorbitol, 2 mM KH_2PO_4, 50 mM KCl, 10 mM $MgCl_2$, 5 mM L-methionine, 0.1% (w/v) fatty acid-free bovine serum albumin, and 50 mM HEPES-KOH, (pH 7.1). To generate or dissipate the membrane potential across the inner mitochondrial membrane, 2.5 mM NADH or 2 μM valinomycin, in the presence of 2 mM ATP was added to import buffer, respectively. Non-imported precursor was removed by treatment with 0.1 mg/ml proteinase K for 15 min and inactivated with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at 0 °C. Where indicated, mitochondria were treated with 0.1 mg/ml trypsin for 30 min on ice, followed by incubation with soybean trypsin inhibitor at a concentration of 0.4–1.0 mg/ml for 15 min (35).

To chase stage III intermediates, after trypsin treatment, de-energized mitochondria with arrested precursors were resuspended in 0.6 M sorbitol, 2 mM NaH_2PO_4, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM L-methionine, 0.1% (w/v) fatty acid-free bovine serum albumin, 50 mM HEPES-KOH, (pH 7.1), 5 mM NADH, 2 mM ATP and incubated for 2 min at 30 °C. The chase was stopped by adding 2 μM valinomycin, 50 mM KCl, and 5 μM FCCP and incubation on ice.

To generate mitoplasts after import, mitochondria were resuspended at a final concentration of 0.01 mg/ml in 20 mM HEPES, (pH 7.4) for 30 min on ice. Mitoplasts were resuspended in import buffer for 15 min in the presence of 0.1 mg/ml proteinase K. The protease was inhibited with 2 mM PMSF for 10 min at 0 °C. For alkali extraction, pellets were resuspended at 0.1–0.5 mg/ml in 100 mM Na_2CO_3 and incubated for 30 min on ice. Mitochondrial membranes were pelleted by ultracentrifugation at 100,000 × *g* for 30 min at 4 °C. Solubilized samples were separated by 12% (w/v) (SDS-PAGE) or by blue native gel electrophoresis (BN-PAGE).

BN-PAGE—Mitochondrial protein (1 mg/ml) was solubilized in 1.6 mg/ml *n*-dodecylmaltoside, 50 mM NaCl, 10% (v/v) glycerol, 20 mM HEPES-KOH, (pH 7.4), 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF for 30 min on ice. Following centrifugation at 25,000 × *g* for 20 min, solubilized protein complexes were size-separated on a 6–16% linear polyacrylamide gradient gel (33, 36). Following electrophoresis, the gel was destained in 7.5% (v/v) glacial acetic acid and 20% (v/v) methanol for 30 min and dried before exposure.

Ni-NTA Affinity Purification of His-tagged Proteins—After import, a total of 0.2 mg of mitochondrial protein was solubilized in 1.6 mg/ml *n*-dodecylmaltoside, 50 mM NaCl, 10% (v/v) glycerol, 20 mM HEPES-KOH, (pH 7.4), 2.5 mM $MgCl_2$, 20 mM imidazole, 1 mM EDTA, 1 mM PMSF for 30 min on ice. The lysate was cleared by centrifugation at 25,000 × *g* and mixed with 40 μl of a 50% (w/v) Ni-NTA Sepharose bead suspension (Novagen) overnight at 4 °C. The beads were washed three times with solubilization buffer and resuspended in Laemmli buffer for analysis by SDS-PAGE or in solubilization buffer for analysis by BN-PAGE.

Miscellaneous Methods—After drying, both SDS-PAGE and BN-PAGE gels were exposed to a phosphorimager (FUJI Bas station version 1.3) to detect radiolabeled proteins, followed by quantification with AIDA software. The standard (Std) represents 10% of the amount of radiolabeled precursor added in each reaction. ECL Western blotting was performed according to Ref. 33.

RESULTS

Import and Localization of HyAAC1 Is Similar to yAAC1—In order to see whether the addition of a histidine tag fused to the N terminus of yAAC1 affected import and localization of this protein, precursor HyAAC1 (see "Experimental Procedures") was incubated with gradient-purified *S. cerevisiae* mitochondria and compared with yAAC1 (Fig. 1, *A* and *B*). ^{35}S-Labeled *in vitro* synthesized HyAAC1 was imported into intact mitochondria and appeared as a major band at ~30 kDa, similar to yAAC1 (Fig. 1, *A* and *B*). Some minor additional bands seen for

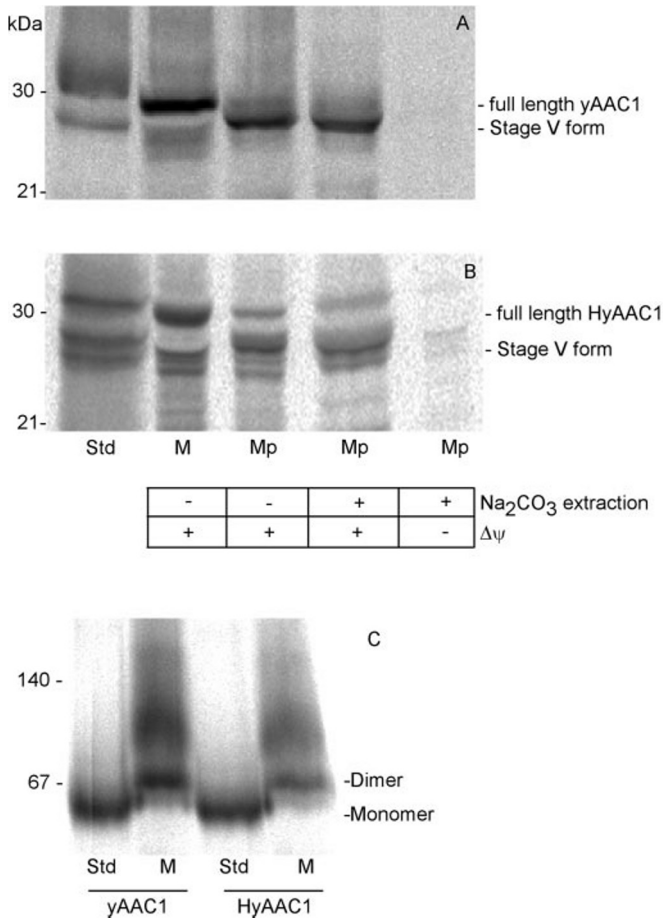


FIG. 1. **Import of wild-type and His-tagged yAAC1.** *In vitro* synthesized yAAC1 (*A*) and HyAAC1 (*B*) were imported into wild-type mitochondria in the presence ($\Delta\psi+$) or absence ($\Delta\psi-$) of a membrane potential, followed by trypsin treatment. After import, mitochondria (*M*) were osmotically shocked to form mitoplasts (*Mp*) in the presence of protease K, the mitoplast pellet extracted with sodium carbonate, and samples analyzed by SDS-PAGE (*A* and *B*) or solubilized for analysis by BN-PAGE (*C*). Bands corresponding to the full-length protein and the protease-resistant fragment are indicated. *Std* represents 10% of the amount of radiolabeled precursor added in each reaction.

the HyAAC1 (*panel B*) compared with yAAC1 (*panel A*) are probably due to the fact that a different promoter (T7 instead of Sp6, see "Experimental Procedures") was used hence leading to internal start sites.

To determine the localization of the imported precursor, mitochondria were converted into mitoplasts and treated with proteinase K. By selectively opening the outer membrane, protease digests any exposed proteins present in the intermembrane space. However, both HyAAC1 and yAAC1 were largely protease protected and resistant to alkali extraction, indicating proper integration into the inner membrane (Fig. 1, *A* and *B*) (31, 32). The slightly lower band that predominates this fraction (*i.e.* stage V AAC) arises from the digestion of both N and C termini protruding into the intermembrane space of fully inserted mature HyAAC1 and yAAC1 (Fig. 1, *A* and *B*).

The proper assembly of oligomers was assayed by BN-PAGE (Fig. 1*C*). Both HyAAC1 and yAAC1 migrated as a major band of ~67 kDa, indicating dimerization of the protein. Higher oligomers were also present but in minor amounts and were seen as diffuse material (probably due to their variable size) at higher molecular weights. When mitochondria were incubated with valinomycin to dissipate the membrane potential prior to import, the monomeric form of HyAAC1 and yAAC1 was predominant (results not shown). HyAAC1 and yAAC1 were unable to integrate into the inner mitochondrial membrane in the absence of a proton motive force confirming that proper assembly into the lipid bilayer is dependent on a membrane potential (37, 38).

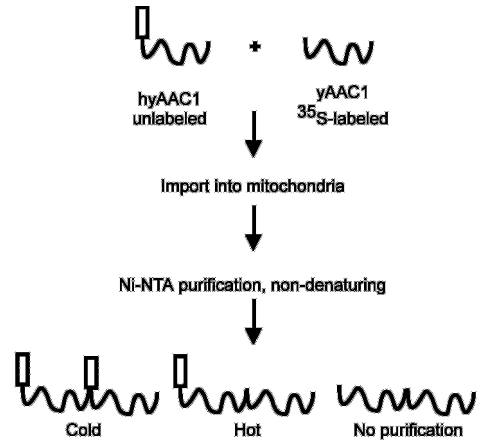


FIG. 2. **Scheme of assay to investigate whether newly imported AAC1 forms a dimer with itself or endogenous AAC.** A 1:1 mixture of HyAAC1 synthesized in the presence of unlabeled methionine and yAAC1 synthesized in the presence of [^{35}S]methionine was imported into mitochondria. Mitochondria were solubilized under non-denaturing conditions using *n*-dodecylmaltoside. The cleared lysate was subjected to affinity purification on Ni-NTA-Sepharose beads.

Nascent AAC1 First Inserts as a Monomer and Then Dimerizes in the Membrane with Endogenous AAC—In order to investigate whether newly imported monomeric AAC1 assembles into a dimer with itself or with endogenous AAC, equal amounts of yAAC1 and HyAAC1 were imported into the mitochondria.

(i) If AAC1 dimerizes with itself prior to import, by importing equal amounts of AAC1 and HyAAC1, dimers could form from: (*a*) two monomers of yAAC1, (*b*) one monomer of AAC1 and HyAAC1, or (*c*) two monomers of HyAAC1. Statistically one would then obtain 25% of *a*, 50% of *b*, and 25% of *c*. To discriminate between yAAC1 and HyAAC1 in this mixture, only the former was ^{35}S-labeled. In this case the only radioactive dimers would be from *a* and *b*, and the only radioactive signal after nickel-affinity purification of detergent-solubilized mitochondria would come from *b*. It would be expected that roughly 50% of the total radioactivity imported would be recovered after Ni-affinity purification.

(ii) In contrast, if newly imported AAC1 or HyAAC1 forms a dimer not with itself but rather with endogenous AAC, then no radioactive signal at all would be obtained after Ni-affinity isolation of detergent-solubilized mitochondria. The above strategy is outlined in Fig. 2.

We performed import experiments for either ^{35}S-labeled HyAAC1 alone (*HyAAC1*), or a mix of equal amounts of cold HyAAC1 and ^{35}S-labeled AAC1 (*mix*), or ^{35}S-labeled AAC1 alone (*AAC1*) (Fig. 3). The assembly of a dimeric species was seen in all three cases following import (hot HyAAC1, hot/cold mix, or ^{35}S-labeled yAAC1 (Fig. 3, *panel A*, *lanes 2*, *6*, and *10*). However, after nickel-affinity purification of solubilized mitochondria, the only radioactive dimeric form recuperated was that from ^{35}S-labeled HyAAC1 imported alone (Fig. 3, *panel B*, *lane 2*). Although large amounts of dimer were formed in the *mix* (Fig. 3, *panel A*, *lane 6*), none of it could be recovered by Ni-affinity purification (Fig. 3, *panel B*, *lane 6*). As a negative control, no Ni-affinity radioactive dimer was isolated when hot AAC1 alone was imported. These data (Figs. 2 and 3) indicate that newly imported AAC1 dimerizes with endogenous AAC and suggest that the endogenous AAC in the membrane must be in a dynamic state that allows dissociation into monomers

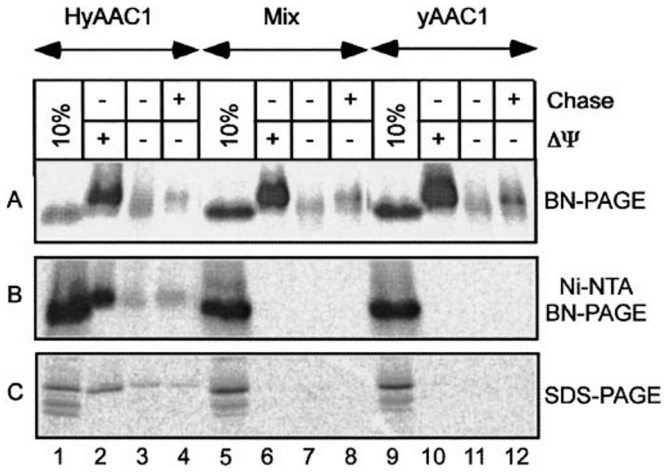


FIG. 3. **Newly imported yAAC1 forms a dimer with endogenous AAC.** *In vitro* synthesized ^{35}S-labeled HyAAC1 or yAAC1 or a 1:1 mixture of unlabelled HyAAC1 and ^{35}S-labeled AAC1 were imported into mitochondria in the presence or absence of a membrane potential ($\Delta\Psi$). Following import, mitochondria were trypsin-treated. Precursors imported into non-energized mitochondria were chased for 2 min (see "Experimental Procedures"). One-quarter of the sample was subjected to BN-PAGE and the rest used for Ni-NTA purification. *A*, analysis of samples by 6–16% BN-PAGE before Ni-NTA purification. Following purification on Ni-NTA Sepharose beads, another quarter of the sample was subjected to BN-PAGE (*B*), and the remainder analyzed by SDS-PAGE (*C*). *Mix*, 1:1 mixture of unlabeled HyAAC1 and labeled yAAC1. 10% represents 10% of the input precursor.

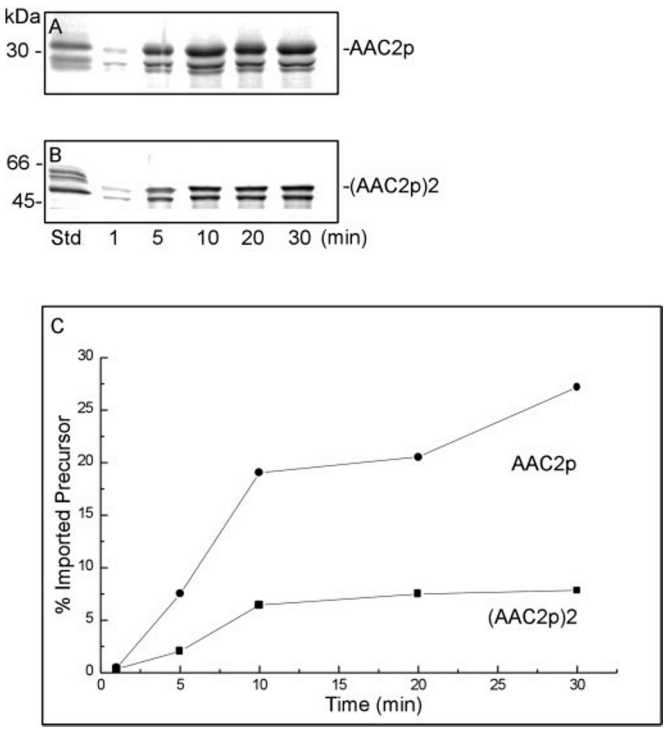


FIG. 4. **Import of monomeric and covalent tandem dimer of AAC2p.** AAC2p (*A*) and $(AAC2p)_2$ (*B*) precursors were imported into mitochondria for varied points in time. Following import, mitochondria were trypsin-treated and extracted using sodium carbonate to separate soluble and membrane fractions, and the pellet subjected to SDS-PAGE. *C*, quantification of AAC2p (*A*) and $(AAC2p)_2$ imported into mitochondria.

that can dimerize with incoming nascent AAC1 monomers.

It could be argued that during the time of import (20 min), newly imported AAC1 may have initially self-dimerized, inserted into the membrane as a dimer, and subsequently dissociated reforming a dimer with endogenous AAC. To investigate this possibility, precursors were arrested at stage III by dissipating the membrane potential for 20 min to have similar amounts of precursor accumulating as in energized mitochondria and then chased by restoring the membrane potential for only 2 min (Fig. 3, all *panels*, *lanes 4*, *8*, and *12*). A dimer formed in all three cases (Fig. 3, *panel A*, *lanes 4*, *8*, and *12*). In this case also, the only radioactive dimer that was isolated by Ni-affinity was that of imported HyAAC1 alone (Fig. 3, *panel B*, *lane 4*). No radioactivity was recovered, even upon overexposure (data not shown), in the *mix*, or after import and chase of AAC1 alone (Fig. 3, *panels B* and *C*, *lanes 8* and *12*). This demonstrates that AAC1 is forming a dimer with endogenous AAC within 2 min after insertion into the lipid bilayer.

Monomeric AAC Inserts More Efficiently Than Dimeric AAC in Organello—To further investigate the native state of AAC at a stage IV form, monomeric (AAC2p), and covalent tandem dimer $(AAC2p)_2$ of isoform 2 was *in vitro* synthesized and imported into wild-type mitochondria (Fig. 4). As expected, the amount of inserted AAC2p and $(AAC2p)_2$ increased with time. After 20 min of import, the amount of AAC2p in the inner mitochondrial membrane was approximately three times higher than $(AAC2p)_2$ (Fig. 4). The fact that *in vivo* the same number of active ATP translocation sites was detected for either the covalent tandem dimer (strain JL1–3-$(AAC2)_2$) or the wild-type strain (34) could be attributed to differences of maturation/stability of the dimer after insertion, or the possibility that some of the endogenous AAC remains in a monomeric state.

An additional experiment was carried out to demonstrate the stage IV form of the adenine nucleotide carrier is monomeric, whereby nascent AAC2p was imported into (JL1–3-$(AAC2)_2$ mitochondria containing only the tandem dimer $(AAC2p)_2$ form (Fig. 5). The formation of dimer was reduced by ~50% at 30 min when AAC2p imported into (JL1–3-$(AAC2)_2$ in comparison to wild-type mitochondria (Fig. 5).

To resolve whether the observed differences in the kinetics of import between the wild type and the JL1–3-$(AAC2)_2$ was due to a difference in endogenous levels of components of the protein translocation machinery, we compared the protein profiles of the two types of mitochondria by Western analysis. (Fig. 6). The AAC2p antiserum detects mainly a polypeptide with an apparent molecular mass of 62 kDa in (JL1–3-$(AAC2)_2$ mitochondria expressing the covalent tandem dimer (34). Some very weak band that would correspond to a monomeric AAC2 is also detected suggesting some degradation of the covalent dimer to a monomer. This could account for some of the dimerization with the incoming monomeric precursor. All other marker proteins tested were present in similar amounts (Fig. 6). Taken together, these data indicate that the difference in kinetics is most likely caused by the inability of the covalently linked dimer form of AAC2 to associate as such with incoming precursor.

The Dimeric Form of yAAC1 Does Not Depend on Intermolecular Disulfide Bonds—There are three conserved cysteine residues in yAAC1 and AACs from other eukaryotes. These residues apparently affect ATP translocation of bovine AAC (39), but it is unknown whether they are also involved in insertion, or dimerization, or both. To test this, an experiment was carried out to see whether dimerization of yAAC1 was due to formation of disulfide bonds between monomers. Following import of yAAC1 into mitochondria, mitoplasts were produced in the presence of proteinase K and either extracted with alkali for SDS-PAGE (Fig. 7*A*) or detergent-solubilized for BN-PAGE (Fig. 7*B*). We found that only a minor proportion of the ~60-kDa species was DTT-sensitive as shown on SDS-PAGE (between 5 and 10% of the total signal). Actually, this DTT-sensitive species proved to be an artifact of the carbonate

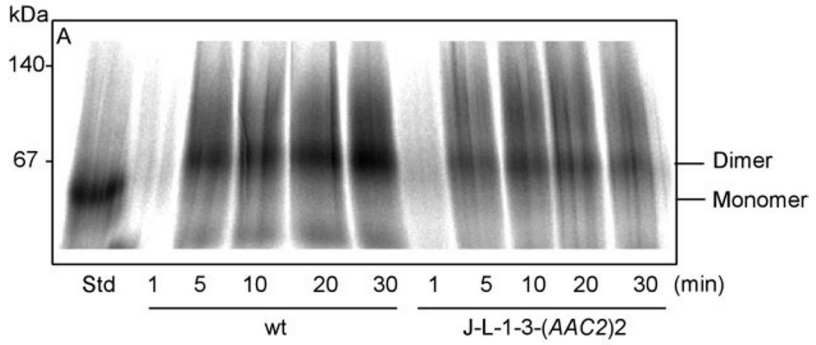
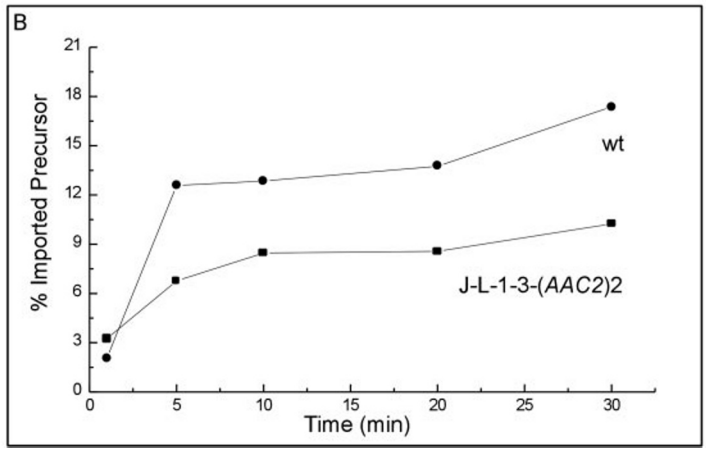


FIG. 5. **Dimer formation in wild-type and JL1–3-$(AAC2)_2$ mitochondria.** *In vitro* synthesized AAC2p was imported into wild-type and JL1–3-$(AAC2)_2$ mitochondria for varied points in time. Following trypsin treatment, mitochondrial samples were analyzed by 6–16% BN-PAGE (*A*). *B*, quantification of AAC2p imported into wild-type and JL1–3-$(AAC2)_2$ mitochondria.



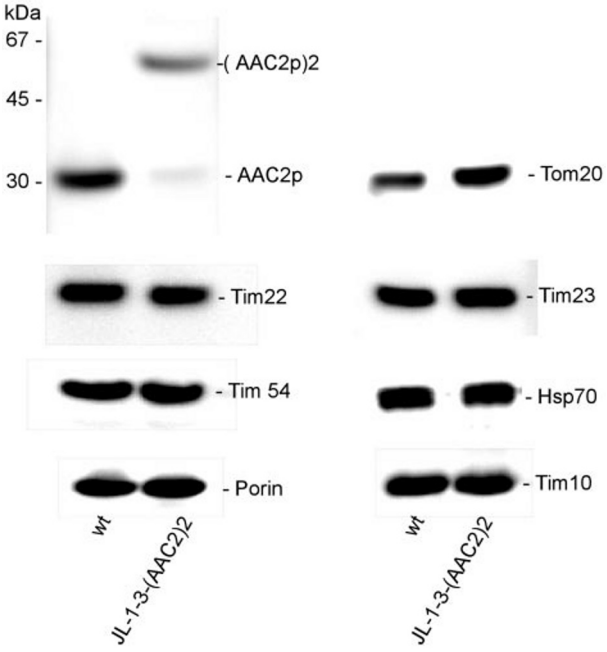


FIG. 6. **Immunodecoration of proteins from wild-type and JL1–3-$(AAC2)_2$ mitochondria.** Fifty micrograms of protein were loaded per lane, resolved by SDS-PAGE, and Western-blotted with antibodies as indicated.

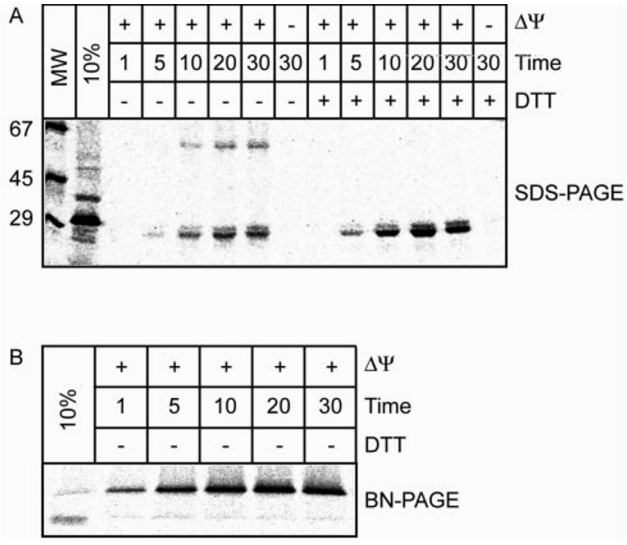


FIG. 7. **The yAAC1 dimer is partially sensitive to DTT.** *In vitro*-synthesized yAAC1 precursor was imported into mitochondria in the presence or absence of ΔΨ for varied points in time. Following import, mitochondria were osmotically shocked to form mitoplasts. This pellet was extracted with sodium carbonate (*A*) and subjected to SDS-PAGE in the presence or absence of DTT or solubilized for analysis by BN-PAGE (*B*).

extraction procedure (data not shown). The vast majority of dimer formed is not dependent on intermolecular disulfide bonds, can already be seen after 1 min of import and increases with time (Fig. 7*B*). These data, in support of our previous results shown in Fig. 3, indicate that dimer formation can occur very fast (less than 1 min shown by BN-PAGE). The assembled yAAC1 dimer does not contain intersubunit disulfide bonds.

Specific Dimerization of yAAC1, but Not Insertion, Is Mediated by Cysteine Residues—To see whether the lack of cysteine residues affects import and dimerization of *in vitro* synthesized yAAC1, as opposed to just mediating intermolecular disulfide bonds in the dimer, cysteine mutants were constructed (see "Experimental Procedures"). All three conserved cysteine residues were mutated to serine residues to create single point mutations or a triple point mutation in yAAC1 (Fig. 8*A*). In

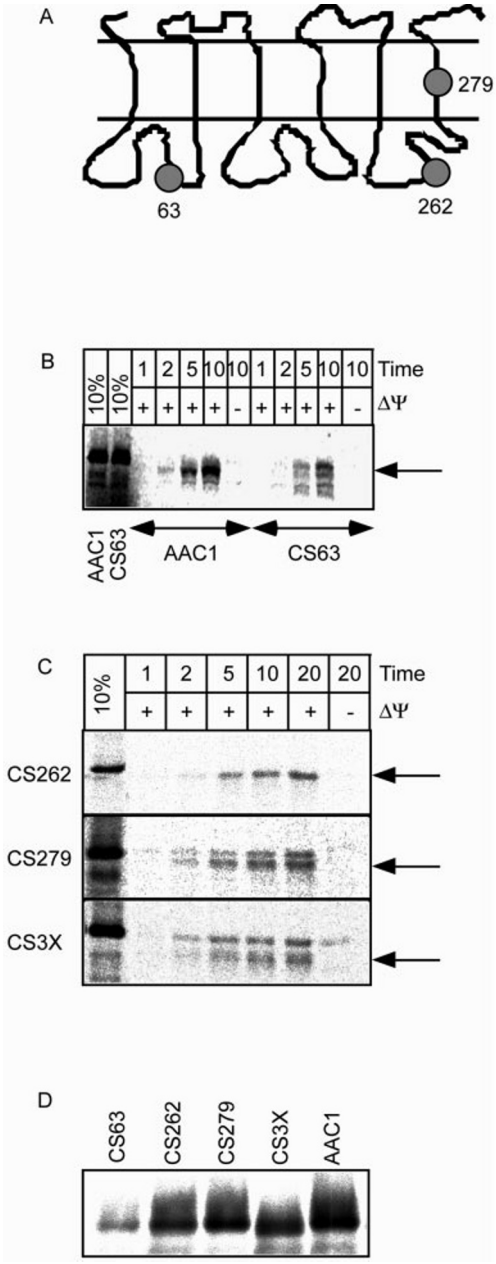


FIG. 8. **Insertion of Cys mutants of yAAC1 into the mitochondrial inner membrane.** *A*, positions of the Cys residues in yeast AAC and mutants generated in this study. *Shaded circles* indicate positions of Cys. Cys-63 and Cys-262 are at equivalent positions in repeats 1 and 3, respectively. *IMS*, intermembrane space; *IM*, inner membrane. *B*, import of CS63 mutant in comparison with yAAC1. *In vitro* synthesized yAAC1 or CS63 was imported into mitochondria in the presence or absence of ΔΨ for varied points in times. Following import, mitochondria were osmotically shocked to form mitoplasts. Pellets were subsequently sodium carbonate extracted and analyzed by SDS-PAGE. *C*, import of C262S, C279S, and C3XS mutants. *In vitro* synthesized Cys mutants were imported into mitochondria in the presence or absence of a membrane potential (ΔΨ) for varied points in time. Following import, mitochondria were osmotically shocked to form mitoplasts. Mitoplasts were sodium carbonate extracted and analyzed by SDS-PAGE. The AAC^{v} (stage V) fragment is indicated by *arrows*. *D*, dimer formation of C63S, C262S, C279S, and C3XS mutants relative to yAAC1. *In vitro* synthesized mutant or yAAC1 protein were imported into isolated mitochondria in the presence or absence of ΔΨ. Following import, mitochondria were osmotically shocked to form mitoplasts. Pellets were solubilized and analyzed by BN-PAGE.

Fig. 8, *B* and *C*, precursor proteins were initially imported into mitochondria, which were subsequently osmotically ruptured to form mitoplasts in the presence of proteinase K, followed by carbonate extraction (to generate stage V). In *panel B*, import kinetics of precursor C63S over a period of 10 min was similar to that observed with yAAC1 indicating that a single point mutation in the first matrix loop connecting the first two transmembrane domains does not significantly affect insertion into the mitochondrial inner membrane. In *panel C*, import kinetics of C262S, C279S, and C3XS were also comparable to C63S. Interestingly, C262S and C279S form a characteristic stage V species of about 29 kDa (*panel C*), similar to yAAC1, while a distinctly lower band of ~26–27 kDa appears upon protease treatment of imported C3XS into the inner mitochondrial membrane (*panel B*). The 10% input precursors for yAAC1 and mutant AACs migrated similarly, indicating that the difference in migration of the cysteine mutants is not due to some initial folding effect of the mutant protein prior to import, but is rather specifically related to mitochondrial insertion. Based on SDS-PAGE analysis (Fig. 8, *B* and *C*) the kinetics of import are similar, with some differences in the pattern of ^{35}S-labeled protein bands of the cysteine mutants compared with yAAC1 presumably due to differences in proper insertion. However, clear differences become apparent when mitoplast samples were analyzed by BN-PAGE (Fig. 8*D*). C63S and C3XS show a significant decrease in dimer formation with about 75 and 50% reduction, respectively compared with the yAAC1. Single point mutations C262S and C279S on the other hand, only result in a 10–30% reduction of dimeric species.

Interestingly, the dimer of the triple cysteine mutant migrates at a lower molecular size compared with that of yAAC1 and all other single point mutations (Fig. 8*D*). It is possible that the triple cysteine mutant does not form a dimer but rather an extended monomer that migrates slightly lower than the wild-type dimer. Alternatively, this mutant protein does form a dimer with a properly inserted endogenous wild-type AAC subunit, but is itself not completely inserted and sticking out of the membrane, hence resulting in a species that migrates faster than a wild-type dimer.

DISCUSSION

The combination of: (i) import of a mixture of cold HyAAC1 and hot AAC1 followed by purification of solubilized mitochondrial extracts on nickel-affinity beads, (ii) comparison of the import of a monomeric (AAC2p) and tandem dimeric species $(AAC2p)_2$, and (iii) import of AAC2p into JL1–3-$(AAC2p)_2$ mitochondria, expressing the covalent tandem dimer of all show that newly inserted monomeric AAC, or stage IV AAC, associates with endogenous AAC to form the stage V dimeric species.

This interaction is dynamic and the formation of a stable stage V dimer depends on the amount of pre-existing, membrane associated monomeric AAC. The dimer is subsequently stabilized by the substrates ADP and ATP. It is generally accepted that dimerization is a prerequisite for the function of AAC or other metabolite carriers (40–44). Alternative models of the functional unit as either monomeric (45) or tetrameric carrier (46) have been proposed, but most available data support the dimeric form as the functional state. In this respect, three groups have recently reported that a covalently linked tandem AAC dimer has identical topology, and very similar biochemical and functional properties to the wild-type noncovalent dimeric AAC (34, 47), although cells harboring the tandem dimer grow slower than wild type (34, 48). These studies supported the idea of a single central channel made up of two associating monomers in a cooperative manner for the transport of ADP and ATP. Our data on the defective insertion of the tandem molecule could explain the slower growth rates (49).

We demonstrate that the majority of the dimer is not sensitive to DTT (Fig. 7*B*) (32). Only 5–10% of the total dimeric form

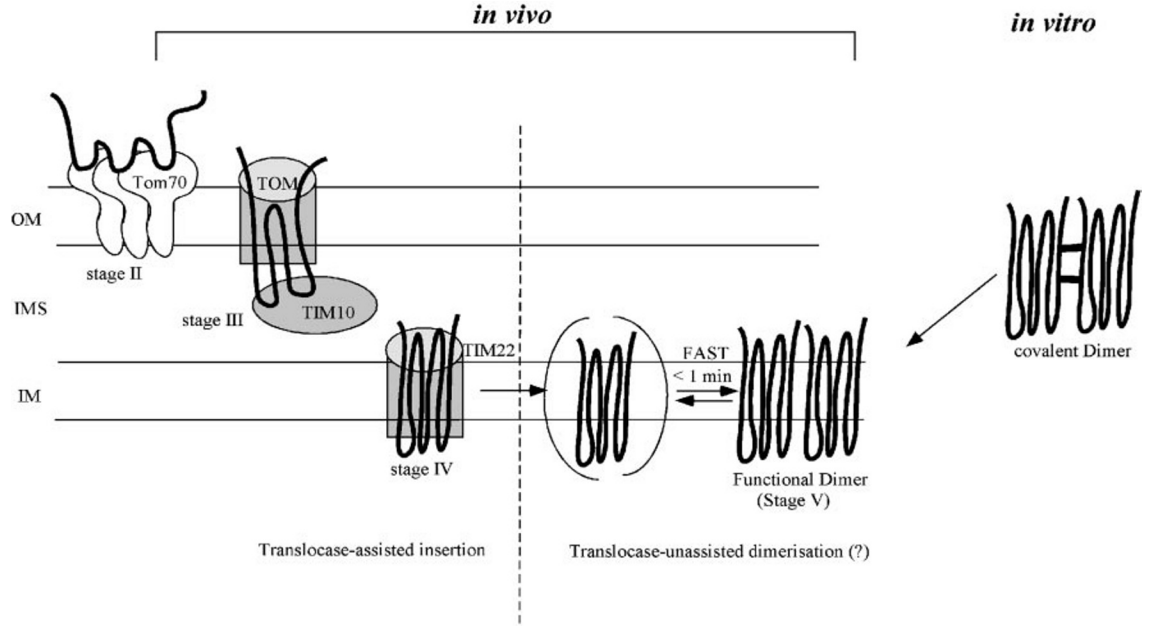


FIG. 9. **Model for insertion and dimerization of carrier proteins.** The TIM machinery interacts with monomeric AAC, and inserts the monomer in a $\Delta\psi$-dependent manner in intermediate stage IV. Subsequent dimerization is guided mainly by monomer-monomer and monomer-CL interactions, and may proceed independently of $\Delta\psi$ and the TIM machinery. The dimerization process is fast, occurring within less than 1 min. Stage IV intermediate forms a dimer with an endogenous AAC monomer dissociating from a dimer (shown in parenthesis). Insertion of preformed covalent dimers of AAC in reconstituted systems *in vitro* does not require the TIM machinery, whose physiological substrate is monomeric AAC (see text for details).

is DTT-dependent and only after carbonate extraction; this could therefore be attributed to subfractionation of the intact mitochondria, in agreement with previous reports where disulfide-bonded (43) or dimaleimide-induced dimers (49) of bovine AAC1 are present in submitochondrial particles, but not in intact mitochondria. Although the final dimeric AAC is independent of intermolecular disulfide bonds, we found that the amount of dimer formed is affected (Fig. 8) when cysteines were mutated to serines (up to 75% reduction in C63S, and 50% reduction in the triple cysteine mutant (Fig. 8*D*). This suggests that binding and/or interactions between cysteine mutants of AAC are decreased in the stage V dimer. The presence of a lower molecular mass of dimeric C3XS (Fig. 8*C*) suggests its extent of insertion into the inner membrane differs markedly from yAAC1 and all single point cysteine mutations (Fig. 8*D*). Most likely the N- and/or C-termini of CS3X are more exposed to the intermembrane space and hence more readily cleaved upon protease treatment of mitoplasts thus producing a lower molecular mass stage V form (Fig. 8*D*). The effect of the cysteine residues on the dimerization process during import is new and had not been previously addressed. As the cysteines are conserved, the effect of the mutation is most likely a direct effect of the chemical nature of the cysteine residue and not related simply to the position of the mutation.

How is this related to the function of AAC? Previous cysteine mutagenesis studies had reported that either single point cysteine to serine mutants (50, 51) or a cysteine-less derivative of yAAC2 (52) could grow on glycerol, indicating these AAC mutants are functional. However, isolation and reconstitution of the C73S yAAC2 mutant (equivalent to our C63S yAAC1 mutant described here) was inactive in transport of ADP/ATP, had much weakened cardiolipin (CL) binding and displayed an absolute requirement for cardiolipin in transport activity (39). In agreement with these findings, Beyer and Nuscher (53) reported that CL binding specifically interferes with labeling of bovine heart Cys-56 (equivalent to yAAC1 Cys-63 and yAAC2 Cys-73). The same Cys-56 had been shown to be important since alkylation inhibited its activity (54). Taken together these data strongly suggested an important role for the first matrix loop cysteine of AAC and a tight interaction with CL (6 molecules CL/AAC dimer see Ref. 55) in transport activity.

Our data show that the functionally impaired cysteine mutants have an additional, specific role in the dimerization of AAC following monomer insertion. The impaired interaction of the mutant monomers with CL may be crucial for the defective dimerization. This possibility is supported by Jiang *et al.* (56) that have shown a weakened AAC dimerization in a yeast cardiolipin mutant. However, since the membrane potential is also affected in this mutant (and hence the insertion of the monomer), a direct effect of CL on the dimerization cannot be unambiguously concluded. Another possibility is that the interaction of the mutant protein either between individual monomers or between a monomer and other protein(s) (for example components of the TIM22 complex) may be affected. Import of AAC into the inner mitochondrial membrane has been divided into five distinct stages (8). In the present study we contributed to the import pathway by demonstrating that monomeric AAC1 is the stage IV intermediate. This can only form a dimer with a pre-existing monomeric AAC, but not with another newly inserted stage IV intermediate. This dimerization process was shown to occur very fast, within less than 1 min, and is affected specifically by cysteine residues. Based on these data we propose the following model (Fig. 9).

After AAC is released from the TOM receptor (stage II), the TIM10 complex recognizes monomeric AAC and associates with it (stage III). At this stage, AAC is delivered to TIM22 and in a membrane potential-dependent reaction it inserts into the inner membrane pore (stage IV). In an elegant recent study, Rehling *et al.* (57) have isolated a stage IV intermediate at relatively low potential levels (60–80 mV) for two other carriers. BN gel analysis showed, in agreement with our data here for AAC, that stage IV is a monomer. Blocking dimerization as in our experiments is an alternative method of isolating this so far elusive intermediate. We have shown that dimerization is guided predominantly by interactions between AAC monomers and possibly AAC monomer-CL interactions. Such interactions

are probably sufficient to provide the energy for dynamic AAC dimer association. In this model, the TIM10/TIM22 complexes would only engage to insert a monomer AAC in a process energetically driven by the membrane potential. Insertion of a stage IV intermediate would be the rate-limiting step, with subsequent dynamic dimerization and dissociation occurring very fast. Insertion of covalently linked dimeric AAC or when dimerization is artificially introduced by disulfide bonding *in vitro*, may indicate some intrinsic capacity of the dimer to insert as such in lipids. It remains open whether the specific dimeric state of such a construct modifies or circumvents some of the TIM10 and TIM22 interactions. *In vivo*, the TIM machinery appears to have evolved to deal with the challenge of monomeric AAC as a substrate: (i) keeping it soluble in the IMS and (ii) inserting it into the inner membrane facilitating thus this rate-limiting step of the AAC assembly to a functional molecule.

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