The EMBO Journal Vol.16 No.15 pp.4560–4567, 1997

Reconstitution of the protein insertion machinery of the mitochondrial inner membrane

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We have reconstituted the protein insertion machinery of the yeast mitochondrial inner membrane into proteoliposomes. The reconstituted proteoliposomes have a distinct morphology and protein composition and correctly insert the ADP/ATP carrier (AAC) and Tim23p, two multi-spanning integral proteins of the mitochondrial inner membrane. The reconstituted system requires a membrane potential, but not Tim44p or mhsp70, both of which are required for the ATP-driven translocation of proteins into the matrix. The protein insertion machinery can thus operate independently of the energy-transducing Tim44p–mhsp70 complex.
Keywords: membrane protein insertion/mitochondria/reconstitution/translocase/yeast

Introduction

The import of nuclear-encoded mitochondrial proteins into the mitochondrial inner membrane or into the matrix is catalyzed by two independent, dynamically interacting translocation systems, the Tom system in the outer and the Tim system in the inner mitochondrial membrane (Horst *et al.*, 1995; for review, see Lill *et al.*, 1996; Haucke and Schatz, 1997; Pfanner and Meijer, 1997). Precursor proteins are recognized by the multi-subunit protein import receptor (Lithgow *et al.*, 1995; Haucke *et al.*, 1996; Lill and Neupert, 1996) and then transferred into and across the mitochondrial outer membrane. Further transport into the mitochondrial inner membrane or into the matrix requires an electrochemical potential across that membrane (Pfanner and Neupert, 1985; Martin *et al.*, 1991) which facilitates the interaction of the incoming precursor chain with the Tim machinery (Bauer *et al.*, 1996). Purified mitochondrial inner membrane vesicles can import mitochondrial precursor proteins with the same efficiency and essentially the same characteristics as intact mitochondria, suggesting that the Tim system can operate independently of the Tom system (Hwang *et al.*, 1989).

Biochemical and genetic studies have identified Tim44p (Maarse *et al.*, 1992; Scherer *et al.*, 1992), Tim23p (Dekker *et al.*, 1993; Emtage *et al.*, 1993) and Tim17p (Maarse *et al.*, 1994; Ryan *et al.*, 1994) as subunits of the Tim translocase. Tim23p and Tim17p are believed to be the core elements of a putative protein-conducting channel (Berthold *et al.*, 1995; Blom *et al.*, 1995). In addition, the N-terminal hydrophilic domain of Tim23p may act as a sensor which recognizes positively charged presequences as they emerge at the the *cis* side of the inner membrane (Bauer *et al.*, 1996). Complete protein translocation into the matrix additionally requires the ATP-dependent action of mitochondrial hsp70 (mhsp70) and mitochondrial GrpE (mGrpEp, also termed Mge1p; Bolliger *et al.*, 1994; Laloraya *et al.*, 1994) which are recruited to the Tim channel via Tim44p, a peripheral protein of the mitochondrial inner membrane (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). Recently, two new components of the Tim machinery, Tim11p and Tim22p, have been identified. Tim11p interacts efficiently with the hydrophobic sorting signal of intermembrane space-targeted precursor proteins (Tokatlidis *et al.*, 1996) and Tim22p appears to be required for the insertion of the ADP/ATP carrier (AAC) family of proteins into the inner membrane and has been reported to be part of a high molecular weight complex distinct from the Tim17p–Tim23p subcomplex (Sirrenberg *et al.*, 1996).

While the mechanism of protein translocation into the mitochondrial matrix has been investigated in some detail (for review, see Lill *et al.*, 1996; Haucke and Schatz, 1997; Pfanner and Meijer, 1997), much less is known about how proteins are inserted into the mitochondrial inner membrane, i.e. conflicting results have been obtained as to whether mhsp70 and ATP are required for this process (Ostermann *et al.*, 1990; Wachter *et al.*, 1992).

In order to understand the process of membrane protein insertion into the mitochondrial inner membrane in more detail, we have reconstituted the protein insertion machinery of the mitochondrial inner membrane into proteoliposomes. The reconstituted vesicles catalyze the insertion of two multi-spanning proteins into their authentic locations within the membrane and require a membrane potential, but not Tim44p or mhsp70. Our results suggest that the inner membrane protein insertion machinery can operate independently of the energy-transducing Tim44p–mhsp70 complex.

Results

Membrane insertion of the ADP/ATP carrier into inner membrane vesicles can be driven by proton pumping or a chlorate diffusion potential

Unlike protein translocation across the endoplasmic reticulum or the bacterial inner membrane (Rapoport *et al.*, 1996), protein import into or across the mitochondrial inner membrane requires an electrochemical potential across that membrane. We therefore first sought a method to generate a potential independent of cytochrome *c* oxidase activity, and then checked whether this potential

is sufficient to drive the insertion of a multi-spanning inner membrane protein such as the AAC.

When the radiolabeled *in vitro*-synthesized precursor of the AAC is imported into inner membrane vesicles, its molecular weight remains unchanged, but a 29 kDa fragment becomes inaccessible to externally added proteinase K (Rassow and Pfanner, 1991). Generation of this resistant fragment (termed AAC′) is abolished upon collapsing the electrochemical potential across the vesicle membrane by K^+ in combination with the K^+-ionophore valinomycin (Rassow and Pfanner, 1991). As the AAC′ fragment is also generated from endogenous, unlabeled AAC in the inner membrane (Wachter *et al.*, 1992), we used it as an indicator for correctly inserted, functional AAC. During import experiments with inner membrane vesicles from yeast mitochondria, correct import of AAC could be driven by the proton-pumping activity of cytochrome *c* oxidase (Figure 1A, $\Delta\mu H^+$-driven import). Alternatively, import could be driven by a chlorate diffusion potential (Poolman *et al.*, 1983) which allows the generation of an electrochemical potential due to the differential diffusion of membrane-permeable chlorate anions and membrane-impermeable sodium cations (Figure 1A, Chlorate-driven import). When the process was driven by a proton gradient (Figure 1A, $\Delta\mu H^+$-driven import), it was inhibited by the protonophore carbonylcyanide *m*-chlorophenylhydrazone (+CCCP) as well as by K^+/valinomycin (+Val). If insertion was driven by a chlorate diffusion potential (Figure 1A, Chlorate-driven import), it was insensitive to CCCP (+CCCP), but sensitive to the K^+/valinomycin (+Val). The chlorate diffusion potential appeared to be stable over at least 10 min since increasing amounts of the AAC were inserted into a protease-protected location over time (Figure 1B).

Generally, insertion of the AAC into inner membrane vesicles appeared to be less efficient than import into intact mitochondria (not shown), presumably reflecting the fact that proteins in the cytosol, such as mitochondrial import-stimulating factor (MSF), or in the intermembrane space contribute to the efficient targeting and delivery of precursor proteins to the mitochondrial surface (for review, see Lill and Neupert, 1996; Haucke and Schatz, 1997).

We have used the diffusion potential-dependent generation of the 29 kDa protease-resistant AAC′ fragment as a functional assay for reconstituting the insertion process from detergent-extracted inner membrane vesicles.

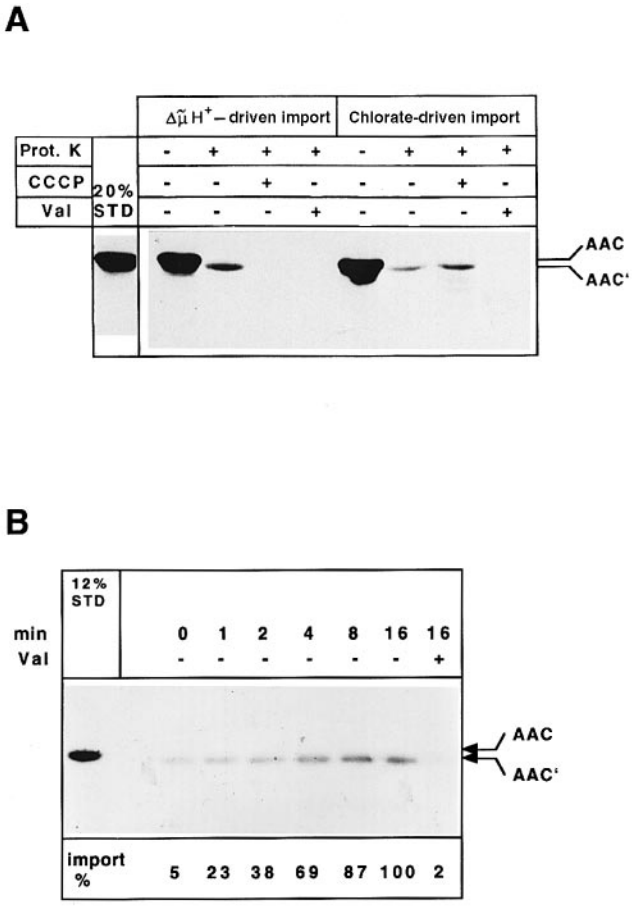

Fig. 1. Membrane insertion of the ADP/ATP carrier into inner membrane vesicles can be driven by a chlorate diffusion potential. (**A**) Inner membrane vesicles (10 μg) were incubated first for 4 min at 30°C in the presence or absence of 10 μM valinomycin (Val) or of 10 μM CCCP (a protonophore), and then for 10 min at 30°C with *in vitro*-synthesized ^{35}S-labeled AAC precursor, either in the presence of 0.6 mg/ml cytochrome *c* and 4 mM ascorbate ($\Delta\mu H^+$-driven import), or in the presence of 50 mM $NaClO_3$ (Chlorate-driven import). Samples were treated for 30 min with 50 μg/ml proteinase K (+ Prot. K) or left untreated (– Prot. K) and then analyzed by SDS–PAGE and fluorography. 20% STD = 20% of the radiolabeled AAC added to each assay. (**B**) Inner membrane vesicles (10 μg) were incubated first for 4 min at 30°C in the presence or absence of valinomycin (Val) and the *in vitro*-synthesized ^{35}S-labeled AAC precursor, followed by a second incubation at 30°C for various times in the presence of 50 mM $NaClO_3$. Samples were treated with 50 μg/ml proteinase K and analyzed by SDS–PAGE and fluorography. 12% STD = 12% of the radiolabeled AAC added to each assay. Radioactive bands were quantified with a β-imager and the amount of AAC′ detected after 16 min was taken as 100%.

The protein insertion machinery of the mitochondrial inner membrane can be reconstituted from detergent extracts of solubilized inner membrane vesicles

When inner membrane vesicles were solubilized in the presence of cholate and yeast mitochondrial lipids, subsequent removal of the detergent by dialysis yielded reconstituted vesicles (Figure 2A) which could insert the radiolabeled precursors of the AAC or Tim23p into their authentic locations (see Figure 3). The reconstituted vesicles had a similar protein composition to purified inner membrane vesicles, as shown by silver staining, except that a few protein bands were missing from the reconstituted vesicles (Figure 2B). Immunoblot analysis revealed that the reconstituted vesicles contained the inner membrane proteins Tim23p and the AAC, but neither the peripheral inner membrane protein Tim44p nor a matrix enzyme, such as the large α-ketoglutarate dehydrogenase subunit (αKDH) (Figure 2C). Compared with inner membrane vesicles, the reconstituted vesicles contained reduced levels of mhsp70 and no detectable mitochondrial processing protease (MPPα/β) (not shown). The negatively stained reconstituted vesicles appeared as a heterogenous population of unilamellar vesicles with diameters ranging from 80 to 200 nm (Figure 2D, right); in contrast, inner membrane vesicles had diameters of 150–400 nm (Figure 2D, left).

The reconstituted vesicles were active in inserting membrane proteins into their authentic locations within

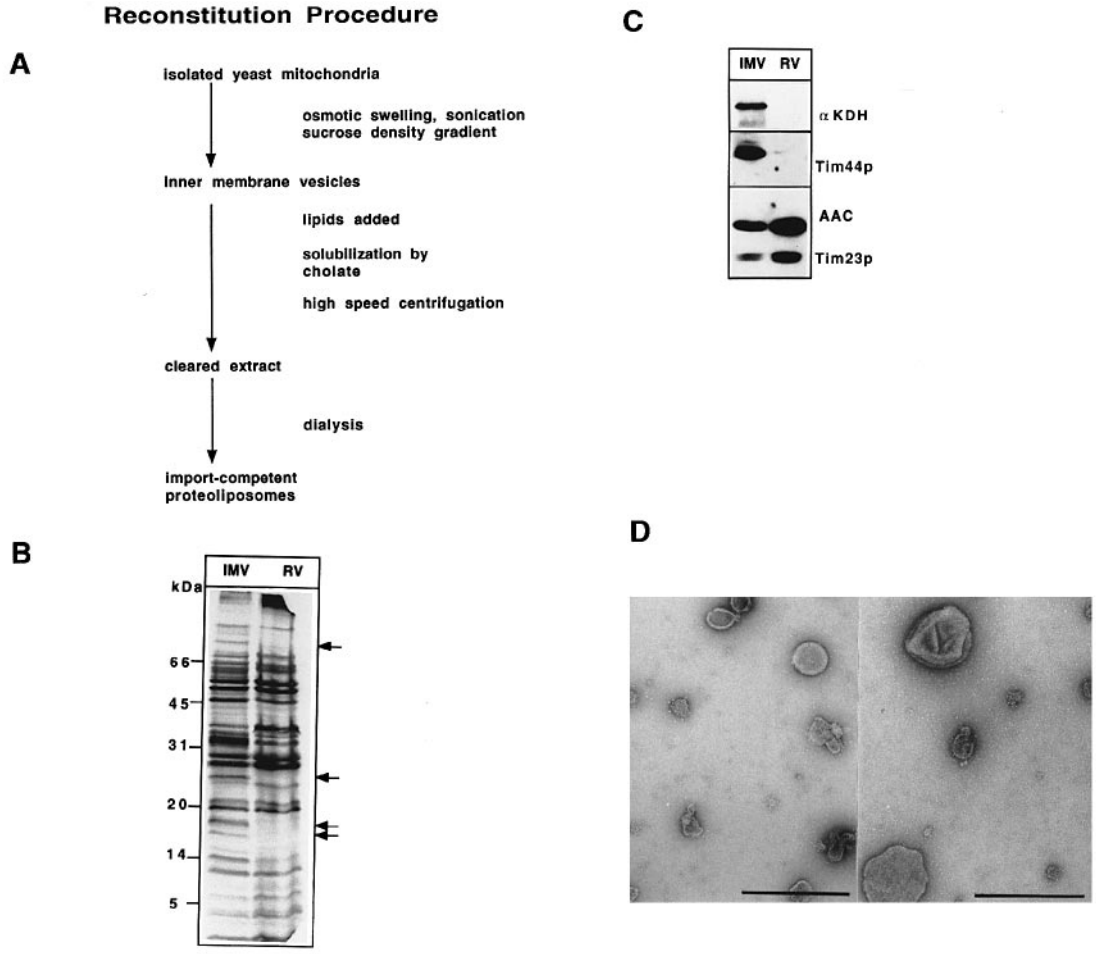

Fig. 2. Reconstitution of insertion-competent proteoliposomes from detergent-solubilized inner membrane vesicles. (**A**) Schematic representation of the reconstitution procedure. See Materials and methods for details. (**B**) Twenty μg of inner membrane vesicles (IMV) or reconstituted vesicles (RV) were analyzed by SDS–PAGE and silver staining. (**C**) Forty μg of IMV or RV were analyzed by SDS–PAGE and immunoblotting with antisera monospecific for the large α-ketoglutarate dehydrogenase subunit (αKDH), Tim44p, the ADP/ATP carrier (AAC) or Tim23p. Immunoblots were developed with [^{125}I]protein A and radioautography. (**D**) Electron micrographs of inner membrane vesicles (left) and reconstituted proteoliposomes (right). Samples were negatively stained with uranyl formate and viewed at a magnification of ×31 500. Bar, 460 nm.

the membrane based on several criteria (Figure 3): like the original inner membrane vesicles, the reconstituted vesicles were able to import the AAC into a protease-protected location within the membrane (Figure 3A). Import was dependent on the addition of chlorate to generate a membrane potential (not shown) and was inhibited by valinomycin (Figure 3A, RV + Val); the inserted AAC generated the 29 kDa AAC′ fragment upon exposure to proteinase K, and the imported AAC was resistant to extraction at pH 11.5 (Figure 3B). Similar results were obtained for the precursor of the inner membrane protein Tim23p (Figure 3C and D): upon the potential-dependent insertion of Tim23p, exposure to trypsin generated a 12 kDa fragment (Tim23p′) (Figure 3C, RV + or – Val). The same fragment is also generated from endogenous Tim23p by trypsin treatment of mitochondria whose outer membrane has been ruptured (mitoplasts; Kübrich *et al.*, 1994). The membrane-inserted Tim23p was resistant to extraction at pH 11.5 (Figure 3D). The efficiency of insertion for both precursors varied generally between 40 and 100% of that observed with inner membrane vesicles when the activity was normalized to the amount of Tim23p, an integral 23 kDa subunit of the inner membrane import machinery (Dekker *et al.*, 1993; Emtage and Jensen, 1993; Berthold *et al.*, 1995).

Unlike inner membrane vesicles, the reconstituted vesicles could not catalyze the complete translocation of the precursor of SU9–DHFR [a fusion protein between the presequence of subunit 9 of the F_0F_1-ATPase from *Neurospora crassa* and mouse dihydrofolate reductase (DHFR); Pfanner *et al.*, 1987] which is transported into the matrix of intact mitochondria and into the lumen of inner membrane vesicles (Figure 3E, IMV). Import of SU9–DHFR into the lumen of inner membrane vesicles is dependent on a membrane potential and resulted in the generation of the fully processed mature form of the protein (Figure 3E, IMV). If the import experiment was performed with reconstituted vesicles, no potential-dependent import or cleavage of SU9–DHFR to the mature form was seen, although we detected a small amount of protease-resistant DHFR fragment (Figure 3E, RV). Since less of this fragment was observed in the absence of a membrane potential, part of it might have resulted from the cleavage of specifically bound or partially translocated SU9–DHFR precursor molecules by externally added proteinase K (Figure 3E, RV).

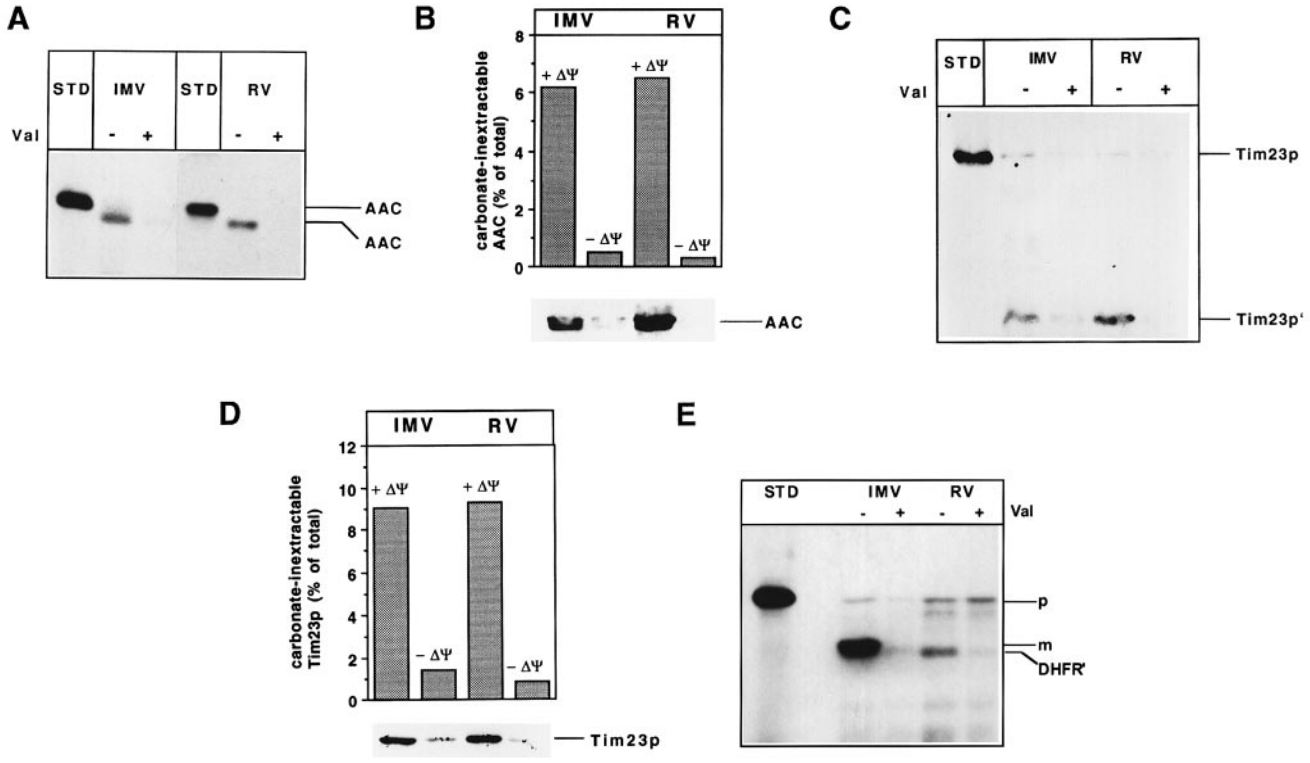

Fig. 3. Reconstituted vesicles catalyze the potential-dependent insertion of the AAC and Tim23p into the membrane. (**A**) Reconstituted vesicles are able to insert the AAC into its authentic protease-protected location within the membrane. Ten μg (protein basis) of inner membrane vesicles (IMV) or reconstituted vesicles (RV) were incubated first for 4 min at 30°C in the presence or absence of the K^+-ionophore valinomycin, and then for 10 min at 30°C with *in vitro*-synthesized ^{35}S-labeled precursor of the AAC and 50 mM $NaClO_3$ (Poolman *et al.*, 1983). Samples were treated with 50 μg/ml proteinase K, re-isolated and analyzed by SDS–PAGE and fluorography. To achieve a better separation of AAC and AAC′, the gel was run for twice as long as in Figure 1. STD = 10% of the radiolabeled AAC added to each assay. (**B**) Membrane-inserted AAC is resistant to extraction by alkaline pH. Following import of the *in vitro*-synthesized ^{35}S-labeled AAC precursor, the vesicles were re-isolated and treated with 100 mM Na_2CO_3 (pH 11.5); after centrifugation, the pellet was washed and analyzed by SDS–PAGE, fluorography and densitometric quantitation of the bands. Import is given as the percentage of the total amount of precursor added to each reaction. (**C**) Reconstituted vesicles are able to insert Tim23p into its authentic protease-protected location within the membrane. Ten μg (protein basis) of IMV or RV were allowed to import the *in vitro*-synthesized ^{35}S-labeled Tim23p precursor as described in (A). Samples were treated with 10 μg/ml trypsin, re-isolated by ultracentrifugation, and analyzed by SDS–PAGE and fluorography. Tim23p, authentic Tim23p; Tim23p′, protease-resistant (i.e. imported) fragment of Tim23p; STD = 10% of the radiolabeled Tim23p added to each assay. (**D**) Membrane-inserted Tim23p is resistant to extraction by carbonate at alkaline pH. Following import of the *in vitro*-synthesized ^{35}S-labeled Tim23p precursor, the vesicles were re-isolated and analyzed as described in (B). (**E**) Reconstituted vesicles are unable to import the precursor of SU9–DHFR into the lumenal space. Ten μg (protein basis) of IMV or RV were incubated first for 4 min at 25°C in the presence or absence of the K^+-ionophore valinomycin, and then for 10 min at 25°C with *in vitro*-synthesized ^{35}S-labeled precursor of SU9–DHFR and 50 mM $NaClO_3$ (Poolman *et al.*, 1983). Samples were treated with 50 μg/ml proteinase K, re-isolated and analyzed by SDS–PAGE and fluorography. STD = 10% of the radiolabeled pSU9-DHFR added to each assay; p, precursor form of SU9–DHFR; m, fully processed mature form of SU9–DHFR; DHFR′, protease-resistant fragment of DHFR.

IgGs against Tim23p inhibit membrane insertion of the AAC

In order to test whether components of the Tim machinery are required for membrane protein insertion, we performed antibody inhibition experiments. When inner membrane vesicles or intact mitochondria were incubated with increasing amounts of IgG monospecific for Tim23p, we observed a dose-dependent inhibition of AAC insertion into inner membrane vesicles, but not intact mitochondria (Figure 4A). The anti-Tim23p IgGs also strongly inhibited insertion of AAC into inner membrane vesicles or reconstituted vesicles whereas pre-immune IgG or IgG against the outer membrane receptor Tom70p were inactive (Figure 4B, IMV and RV). In contrast, import of AAC into intact mitochondria was inhibited by IgG against Tom70p, but not by IgG against Tim23p (Figure 4B, MITO). Our anti-Tim23p IgG appeared to be monospecific since it decorated only a single 23 kDa band in extracts of wild-type mitochondria and a single 27 kDa band in mitochondria from a strain in which the chromosomal copy of *TIM23* had been disrupted and replaced by a plasmid-borne gene encoding a hexahistidine-tagged version of Tim23p (Figure 4C). Thus, we can exclude the possibility that our anti-Tim23p IgG cross-reacts with another protein of similar size such as Tim22p, a recently identified subunit of the Tim machinery (Sirrenberg *et al.*, 1996). The potential-dependent correct insertion of AAC into the membrane of the reconstituted proteoliposomes thus appears to require Tim23p.

Vesicles reconstituted from Tim23p-depleted, but not from Tim44p- or mhsp70-depleted detergent extracts are unable to insert the AAC correctly

Tim44p (Maarse *et al.*, 1992; Scherer *et al.*, 1992), mhsp70 (Kang *et al.*, 1990; Scherer *et al.*, 1990) and mGrpE (also termed Mge1p or Yge1p; Bolliger *et al.*, 1994; Laloraya *et al.*, 1994; Nakai *et al.*, 1994) are required for the ATP-dependent translocation of preproteins across the mitochondrial inner membrane (Kronidou *et al.*, 1994;

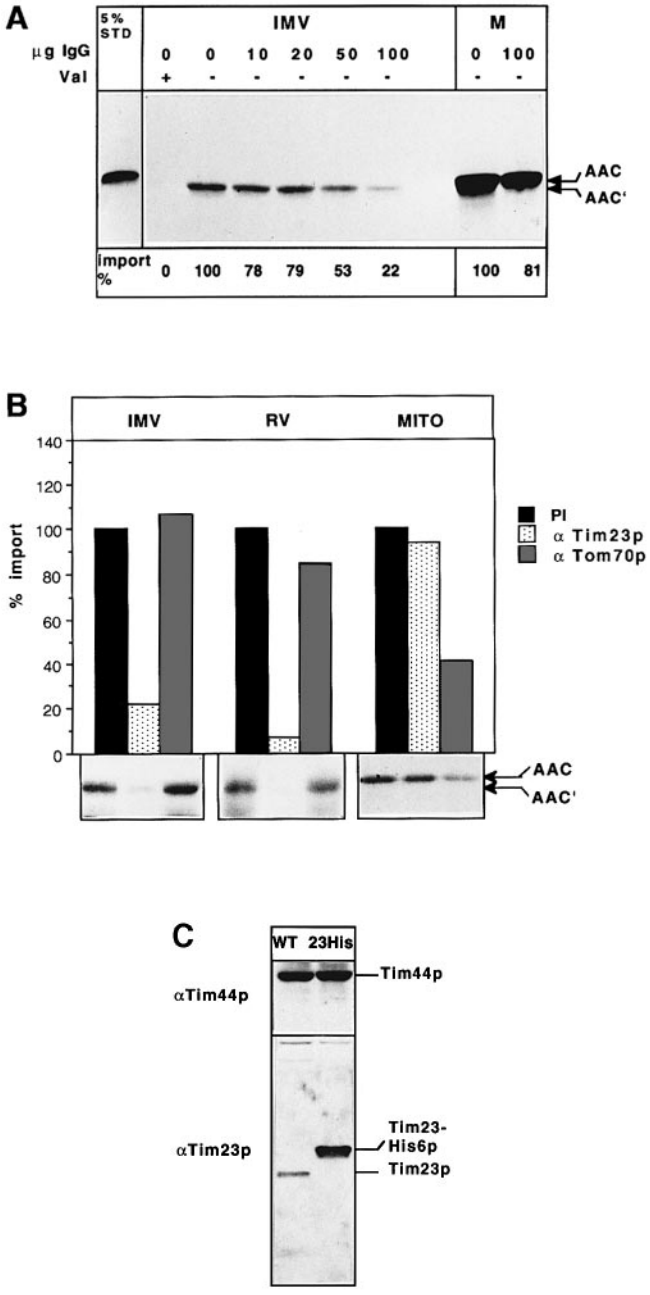

Fig. 4. IgGs against Tim23p inhibit membrane insertion of the AAC. (**A**) Ten µg of inner membrane vesicles (IMV) or intact mitochondria (M) were pre-incubated for 60 min on ice in the presence of the indicated amounts of IgG against Tim23p. Where indicated, valinomycin was added (+ Val) and import was initiated by adding the *in vitro*-synthesized ^{35}S-labeled AAC precursor, cytochrome *c* (to 0.6 mg/ml) and ascorbate (to 4 mM). Import was allowed to proceed for 5 min at 30°C. Samples were treated with 50 µg/ml proteinase K and analyzed by SDS–PAGE, fluorography and densitometric quantification of the bands. The intensity of the band obtained in the absence of IgG was taken as 100%. 5% STD = 5% of the radiolabeled AAC added to each assay. (**B**) Ten µg of IMV, reconstituted vesicles (RV) or mitochondria (MITO) were incubated for 1 h on ice with 80 µg of IgG from pre-immune serum (PI) or from antisera monospecific for the inner membrane protein Tim23p or the outer membrane receptor Tom70p. Import of AAC was performed as described in Figure 3A. Samples were analyzed by SDS–PAGE, fluorography and densitometric quantification of the bands. The intensity of the radioactive band obtained in the presence of pre-immune IgG was taken as 100%. Ordinate: percentage of AAC import relative to mitochondria or to inner membrane vesicles treated with pre-immune IgG. (**C**) The polyclonal anti-Tim23p antiserum is monospecific for Tim23p. Mitochondria isolated from a yeast strain expressing wild-type Tim23p (WT) or from an isogenic strain expressing only a hexahistidine-tagged version of Tim23p (23His) were analyzed by SDS–PAGE and immunoblotting with polyclonal antisera against Tim44p (αTim44p, upper part) or Tim23p (αTim23p, lower part; the autoradiogram of the whole gel is shown). The reaction was developed with the ECL detection kit (Amersham Inc.).

Rassow *et al.*, 1994; Schneider *et al.*, 1994). To test whether these components are also required for the reconstituted insertion sytem, we immunodepleted a detergent extract of solubilized inner membrane vesicles with protein A–Sepharose beads containing pre-immune IgG or IgG monospecific for either Tim44, mhsp70 or Tim23p (Figure 5A). Immunoblotting of the depleted extracts verified that >98% of each cognate antigen was removed by this procedure (Figure 5A). When the depleted extracts were reconstituted into proteoliposomes, the Tim23p-depleted extract was inactive (Figure 5B; α23), whereas the Tim44p-depleted and the mhsp70-depleted extracts were fully active (Figure 5B; α44, α70). As the amounts of Tim44p, mhsp70 (Figure 5B; α23) or the endogenous AAC (not shown) were not affected by immunodepleting Tim23p from the detergent extract, the import defect of proteoliposomes from the Tim23p-depleted extract did not reflect removal of residual intact inner membrane vesicles. Membrane insertion of AAC thus appears to require Tim23p, but not mhsp70 or Tim44p. This result is consistent with the observations that the active vesicles reconstituted from undepleted extracts lack Tim44p (Figure 2C), and that insertion of AAC into the inner membrane of intact mitochondria does not require ATP in the matrix (Wachter *et al.*, 1992). The previously reported effect of a temperature-sensitive mhsp70 mutation on the insertion of AAC into mitochondria (Ostermann *et al.*, 1990) may therefore reflect indirect effects of this mutation.

Discussion

The reconstitution of protein translocation systems from bacteria (Brundage *et al.*, 1990) and the endoplasmic

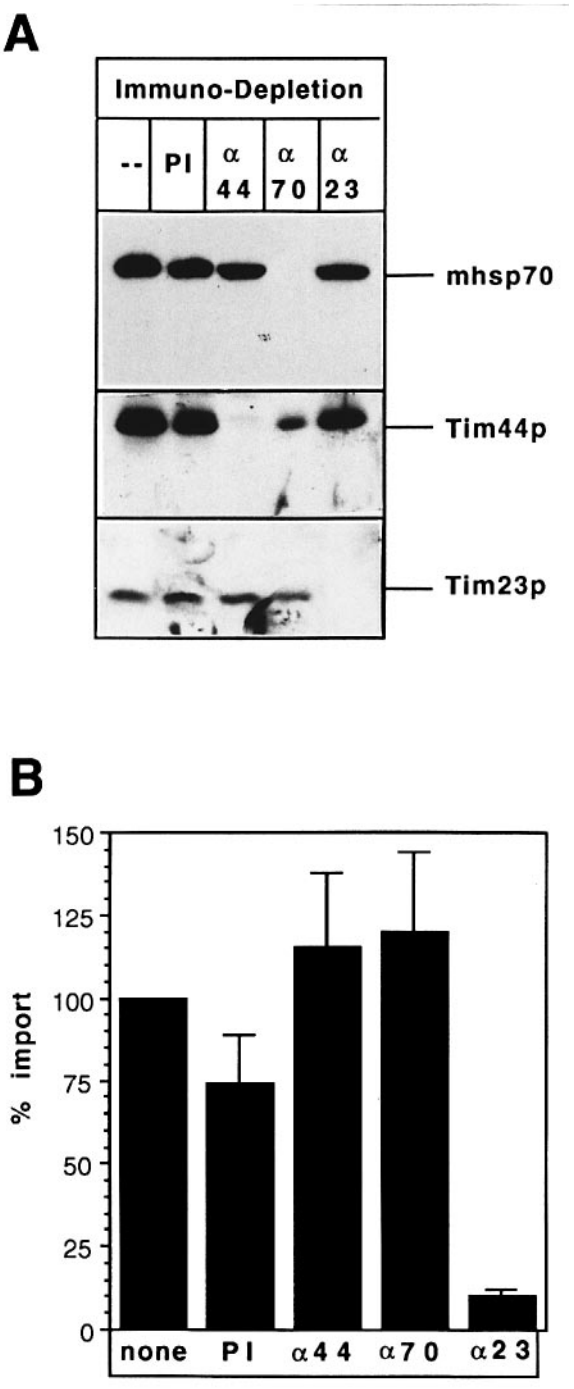

Fig. 5. Depletion of Tim23p, but not of Tim44p or mhsp70 from the detergent extract inhibits reconstitution of insertion-active proteoliposomes. (**A**) Aliquots of a detergent extract (see Figure 2A and Materials and methods) of inner membrane vesicles were either left untreated (none), or depleted of the respective antigen by incubation for 6 h at 4°C with protein A–Sepharose beads carrying IgG from pre-immune serum (PI), or from antiserum monospecific for either mhsp70 (α70), Tim44p (α44) or Tim23p (α23). Each of the five aliquots was then precipitated with TCA and analyzed by SDS–PAGE and immunoblotting with antisera against mhsp70, Tim44p or Tim23p. (**B**) Reconstituted proteoliposomes were made from the five aliquots of detergent extracts described in (A) and analyzed for their ability to import AAC as described in Figure 3A. Ordinate: percentage of AAC imported relative to proteoliposomes prepared from the untreated extract. The data represent mean values from three independent reconstitution experiments. The amount of AAC imported into proteoliposomes prepared from the untreated extract was taken as 100%.

reticulum (Nicchitta and Blobel, 1990; Brodsky *et al.*, 1993; Görlich and Rapoport, 1993; Panzner *et al.*, 1995) into proteoliposomes has given remarkable insight into how these systems work. In the present study, we describe conditions that allow the reassembly of insertion-competent proteoliposomes from detergent extracts of solubililized yeast mitochondrial inner membrane vesicles. The reconstituted system is capable of catalyzing the potential-dependent insertion of membrane proteins into the vesicular membrane and does not require Tim44p–mhsp70 which has an essential role in facilitating the ATP-driven vectorial transfer of preproteins across the mitochondrial inner membrane (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). Recent evidence, however, indicates that Tim44p may not be the only membrane anchor for mhsp70; a Tim23–Tim17p subcomplex appears to provide a second ATP-dependent binding site for mhsp70 (Bömer *et al.*, 1997). Although the physiological role of the Tim23p–Tim17p–mhsp70 complex is not entirely clear, it has been postulated that the two mhsp70-containing subcomplexes cooperate in the import of tightly folded preproteins where a strong pulling force is needed (Pfanner and Meijer, 1997). The data presented here demonstrate that neither of the two subcomplexes participates in the insertion of membrane proteins since reconstituted vesicles devoid of mhsp70 are competent to insert AAC correctly. However, we cannot rule out the possibility that mhsp70 might be required for the 're-priming' of the protein insertion machinery for multiple rounds of import, as suggested for BiP/Kar2p in the yeast endoplasmic reticulum (Brodsky *et al.*, 1995).

We further demonstrate by using two different methods that the reconstituted system, like the purified inner membrane vesicles, requires Tim23p for the insertion activity. First, IgG against Tim23p blocks import of the AAC into inner membrane vesicles and into the reconstituted vesicles. Second, reconstituted vesicles lacking Tim23p are unable to promote the insertion of the AAC.

Based on the results reported here and elsewhere (Dekker *et al.*, 1993; Emtage and Jensen, 1993), we favor a model according to which Tim23p is part of the general translocation machinery in the mitochondrial inner membrane and serves a dual role in the import process: the N-terminal domain functions as a potential-dependent presequence receptor with a particularly important role in the import of matrix-targeted precursors (Bauer *et al.*, 1996), whereas the membrane-spanning region is part of a protein-conducting channel in the mitochondrial inner membrane. In a recent report, Sirrenberg *et al.* (1996) have identified Tim22p, a novel component of the Tim system with homology to both Tim23p and Tim17p which appears to have an essential role in the insertion of carrier proteins such as AAC. It will be interesting to see whether depletion of Tim22p and its putative partner proteins results in a similar inhibition of carrier protein insertion as reported for Tim23p here. The insertion of membrane proteins may therefore be catalyzed by the cooperative action of the Tim23p complex with Tim22p and its putative partner subunits (Sirrenberg *et al.*, 1996), while complete translocation of proteins into the matrix requires the recruitment of mhsp70 to the membrane (either via Tim44p or via Tim17–Tim23p; Bömer *et al.*, 1997)

The development of a reconstituted mitochondrial

import system and the direct demonstration that the protein insertion machinery can operate independently of Tim44p and mhsp70 should allow the further biochemical dissection of the insertion process, and might form the basis for the reconstitution of the complete translocation pathway into the mitochondrial matrix.

Materials and methods

Cell growth and isolation of mitochondria

Experiments except for the one shown in Figure 4C were performed with yeast strain D273-10B (MATα; ATCC 25657). The experiment shown in Figure 4C was carried out with the 'wild-type' yeast strain YRJ436 (MATα *mas6::URA3 leu2 trp1 ura3 his4 cyh2*) transformed with a derivative of pRS314 (*TRP1 CYH2 CEN6*) encoding the wild-type Tim23p (a kind gift of Dr R.E.Jensen, Johns Hopkins University) or strain YHV23 (MATα *mas6::URA3 leu2 trp1 ura3 his4 cyh2*) encoding a hexahistidine-tagged version of Tim23p expressed from the ADHI promoter (*TRP1 CEN6*). Cells were cultured on semisynthetic medium supplemented with 2% sodium lactate and 0.1% glucose. Mitochondria were isolated and purified as described by Glick and Pon (1995).

Reconstituition of proteoliposomes from detergent extracts of solubilized inner membrane vesicles

Yeast mitochondrial inner membrane vesicles were prepared from isolated mitochondria according to Hwang *et al.* (1989). They were solubilized in buffer A [20 mM HEPES-KOH pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 20% glycerol, 0.4 M sorbitol] in the presence of 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% sodium cholate and 1 mg/ml yeast mitochondrial lipids. Mitochondrial lipids were isolated according to Rousser and Fleischer (1967). Insoluble material was pelleted for 20 min at 150 000 *g*. The cleared extract was dialyzed against 100 volumes of buffer B (20 mM HEPES-KOH pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT), for 20 h at 4°C. For import reactions, reconstituted vesicles were pelleted by ultracentrifugation at 150 000 *g* and suspended in import buffer (0.6 M sorbitol, 50 mM HEPES-KOH pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2 mM ATP) at a protein concentration of 0.1 mg/ml. Vesicles reconstituted with a mixture of phosphatidylcholine, phosphatidylethanolamine and cardiolipin from bovine brain (weight ratio 2:1:1), instead of yeast mitochondrial lipids, were equally active in importing AAC into the vesicle membrane (data not shown).

Protein import into mitochondria or membrane vesicles

Precursor proteins were imported into isolated yeast mitochondria as described previously by Wachter *et al.* (1994). Protein import into membrane vesicles was assayed by incubating 10 μg (protein basis) of inner membrane vesicles or reconstituted vesicles for 4 min at 25 or 30°C (see figure legends) in the presence or absence of the K^+-ionophore valinomycin (Val, 10 μM), followed by a second incubation for 10 min at 25 or 30°C with 10 μl of the *in vitro*-synthesized ^{35}S-labeled precursor protein and either 0.6 mg/ml cytochrome *c* and 4 mM ascorbate ($\Delta\mu H^+$-driven import), or 50 mM $NaClO_3$ (Chlorate-driven import) (Poolman *et al.*, 1983). To assay for membrane insertion of imported precursor proteins, inner membrane vesicles or reconstituted vesicles were re-isolated at 150 000 *g*, resuspended at 0.5 mg/ml in 100 mM Na_2CO_3 (pH 11.5), incubated on ice for 30 min and spun at 150 000 *g* at 4°C for 15 min. The pellet was resuspended in an equal volume of 100 mM Na_2CO_3 (pH 11.5); proteins from pellet and supernatant were precipitated with 10% trichloroacetic acid (TCA) and analyzed by SDS–PAGE, fluorography and densitometric quantitation of the bands. To assay for protease protection of imported proteins, the vesicles were re-isolated, resuspended in import buffer containing 50 μg/ml proteinase K or 10 μg/ml trypsin and incubated on ice for 30 min; digestion was stopped by addition of 1 mM PMSF. Total proteins were precipitated with 10% TCA. Samples were analyzed by SDS–PAGE, fluorography and densitometric quantitation of the bands.

Antibody inhibition of protein insertion into mitochondria or membrane vesicles

Ten μg of inner membrane vesicles, reconstituted vesicles or mitochondria were incubated for 1 h on ice with IgG from pre-immune serum or from antisera monospecific for the inner membrane protein Tim23p (a rabbit polyclonal antiserum raised against a β-galactosidase–Tim23 fusion protein) or the outer membrane receptor Tom70p (a rabbit polyclonal antiserum raised against the 60 kDa cytosolic domain of Tom70p). Import of AAC was performed as described above. Samples were analyzed by SDS–PAGE, fluorography and densitometric quantification of the bands.

Immunodepletion of detergent extracts made from solubilized inner membrane vesicles

Aliquots of a detergent extract (see above) from solubilized inner membrane vesicles were either left untreated, or depleted of the respective antigen by incubation for 6 h at 4°C with protein A–Sepharose beads carrying IgG from pre-immune serum (PI), or from antiserum monospecific for either mhsp70 (α70), Tim44p (α44) or Tim23p (α23). The IgG-carrying protein A–Sepharose beads were removed by centrifugation and the supernatant was subjected to another round of immunodepeletion with fresh immunobeads. For these experiments, the gylcerol concentration in the solubilization buffer was reduced to 10% to allow complete removal of the respective antigen during the immunodepletion, resulting in a 20–30% reduced import efficiency of the corresponding reconstituted vesicles. Each of the five aliquots was then precipitated with TCA and analyzed by SDS–PAGE and immunoblotting with antisera against mhsp70, Tim44p or Tim23p.

Miscellaneous

Published methods were used for SDS–PAGE, *in vitro* transcription–translation, silver staining and immunoblotting (Ramage *et al.*, 1993; Wachter *et al.*, 1994). Protein concentrations were determined with the BCA method (Pierce Inc.). Fluorograms were quantified using a computerized β-imager (Biospace Instruments, Paris, France).

Acknowledgements

We thank Dr R.E.Jensen for the kind gift of the Δ*tim23* strain (YRJ436), Dr K.Tokatlidis for the TIM23-His_6 clone, A.Hefti for the electron microscopy, W.Oppliger for excellent technical assistance and Drs A.Driessen, W.Konings, A.Watts and W.Wickner for valuable advice. Finally, we wish to thank Drs K.Tokatlidis and A.Schneider and members of our laboratory for discussions and critical reading of the manuscript. This study was supported by grants from the Swiss National Science Foundation and the Human Capital and Mobility Program of the EEC (to G.S.) and by a fellowship from the Boehringer Ingelheim Fund (to V.H.).

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Received on April 17, 1997; revised on May 16, 1997