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The ΔΨ- and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria

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New steps in the reaction cycle that drives protein translocation into the mitochondrial matrix have been defined. The membrane potential (ΔΨ)- and the mtHsp70/MIM44-dependent import machinery co-operate in the transfer of the presequence across the inner membrane. Translocation intermediates, arrested at a stage where only the presequence could form a complex with mtHsp70, still required ΔΨ for further import. ΔΨ at this stage prevented retrograde movement, since mtHsp70 did not bind to the presequence with sufficient affinity. In contrast, mature regions of incoming chains adjacent to the presequence were bound by mtHsp70 tightly enough to stabilize them in the matrix. Cycling of the mtHsp70 on and off incoming chains is a continuous process in the presence of matrix ATP. Both MIM44-bound and free forms of mtHsp70 were found in association with the incoming chains. These data are consistent with a reaction pathway in which the mtHsp70/MIM44 complex acts as a molecular ratchet on the *cis* side of the inner membrane to drive protein translocation into the matrix.

Keywords: membrane potential/MIM44/mitochondrial protein import/molecular ratchet model/mtHsp70

Introduction

Import of nuclear-encoded proteins into mitochondria is a multi-step process that is facilitated by the coordinated action of different teams of proteins located in the cytosol, mitochondrial membranes and matrix space (Pfanner *et al.*, 1994; Stuart *et al.*, 1994a; Lithgow *et al.*, 1995). Import components present in the cytosol include molecular chaperones of the heat shock protein 70 (Hsp70; Chirico *et al.*, 1988; Deshaies *et al.*, 1988) and DnaJ (Cyr *et al.*, 1994) families as well as import stimulating factors (Hachiya *et al.*, 1993, 1995). Chaperone proteins interact with precursor proteins in a co-translational manner (Beckmann *et al.*, 1990; Frydman *et al.*, 1994) to assure they arrive at the mitochondrial outer membrane (MOM) in a transport-competent conformation (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Caplan *et al.*, 1992). A protein complex in the outer membrane (MOM complex), composed of at least seven different polypeptides, recognizes the mitochondrial targeting signal/presequence and facilitates the binding of precursor proteins to mitochondria (Kiebler *et al.*, 1990; Söllner *et al.*, 1992; Lithgow *et al.*, 1995). The MOM complex is thought to provide a pore that allows passage of the presequences (Kiebler *et al.*, 1990). An import machinery in the mitochondrial inner membrane (MIM) then recognizes the presequence and facilitates its transfer to the matrix (Glick, 1995; Pfanner and Meijer, 1995). Movement of the presequence into the matrix is assisted by MIM17, MIM23 and possibly MIM14 and MIM33 which constitute the MIM complex (Berthold *et al.*, 1995). Import of the presequence is also dependent upon the presence of a membrane potential across the inner membrane (ΔΨ; Schleyer and Neupert, 1985). Upon leaving the MIM import channel, the presequence has been suggested to interact with the peripheral membrane import component MIM44. MIM44 recruits the mitochondrial cognate of Hsp70 (mtHsp70) to the import channel by binding it in a 1:1 complex (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). The mtHsp70/MIM44 complex then drives the completion of import via an ATP-dependent mechanism that has been proposed to be analogous to a 'molecular ratchet' (Schneider *et al.*, 1994).

The molecular ratchet model predicts that spontaneous diffusion of limited segments of precursor polypeptides across mitochondrial membranes (Neupert *et al.*, 1990; Schneider *et al.*, 1994) is possibly due to local unfolding reactions. ATP is consumed by the mtHsp70 component of the mtHsp70/MIM44 complex to bind and trap segments of the incoming chain in the matrix (Cyr *et al.*, 1993; Ungermann *et al.*, 1994). Proteins must be in an unfolded conformation to traverse the mitochondrial membranes (Eilers and Schatz, 1986; Rassow *et al.*, 1990). In spite of the action of cytosolic chaperones, some precursor proteins arrive at the membrane surface with folded subdomains (Glick *et al.*, 1993; Stuart *et al.*, 1994b). MtHsp70 plays a role in the unfolding of these subdomains on the MOM surface (Gambill *et al.*, 1993; Glick *et al.*, 1993; Stuart *et al.*, 1994b). Two proposals have been put forth to explain the unfoldase activity of mtHsp70 (Schneider *et al.*, 1994; Glick, 1995). The first is that binding of mtHsp70 to the incoming chain in the matrix shifts the equilibrium of the folding reaction on the membrane surface to the unfolded state (Schneider *et al.*, 1994). The other is that mtHsp70 utilizes the ATP hydrolysis-dependent conformational change to generate a force that is capable of pulling folded domains on proteins apart (Glick, 1995). The mechanism by which mtHsp70 acts to unfold precursor proteins is likely to reflect the mechanism by which it drives proteins across the inner membrane. Therefore the basic mechanism by which mtHsp70 drives the translocation of proteins into mitochondria is an issue of debate.

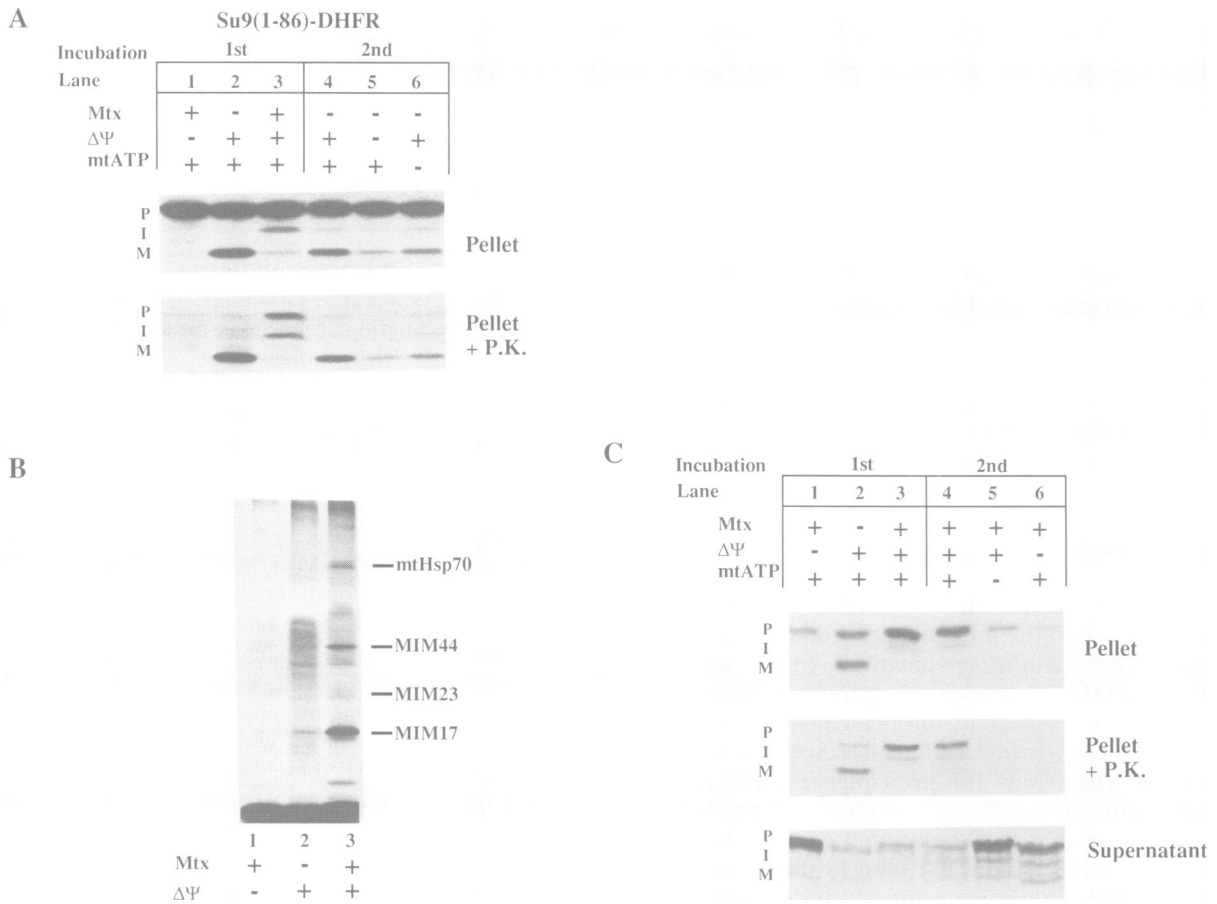

Fig. 1. ΔΨ is required for efficient translocation and stabilization of the presequence during initial steps of import across the inner membrane. (**A**) Chase of Mtx-arrested pSu9(1–86)–DHFR into the mitochondrial matrix. ^{35}S-Labeled pSu9(1–86)–DHFR was incubated in a 100 μl reaction buffer supplemented with 1 mM NADH for 5 min with mitochondria (250 μg/ml). After the initial import reaction, sample 3, which had a 4-fold reaction volume, was split into four equal aliquots. One was left on ice (lane 3); mitochondria from the other three were reisolated, washed with import buffer without Mtx and resuspended in import buffer containing either 2 mM ATP (lane 4), 2.5 μM CCCP and 2 mM ATP (lane 5), or 20 μM oligomycin and 40 U/ml apyrase (lane 6). Samples were then incubated for an additional 20 min at 25°C. Where indicated, samples were treated with proteinase K to digest non-imported proteins associated non-specifically with mitochondria. Analysis of precursor (P) to intermediate (I) and mature (M) forms was done by SDS–PAGE and fluorography (for details see Materials and methods). (**B**) Cross-linking of Mtx-arrested pSu9(1–86)–DHFR to components of the import machinery. Mitochondria were incubated for 5 min at 25°C with ^{35}S-labeled pSu9(1–86)–DHFR in the presence or absence of Mtx. For cross-linking, DSS was added to 200 μM for 30 min at 0°C. Cross-link adducts were identified by immunoprecipitation with antibodies specific for the proteins indicated. For details see Materials and methods. (**C**) Dissipation of ΔΨ causes Mtx-arrested pSu9(1–86)–DHFR to slide out of the MIM import apparatus. Import reactions were performed for 2.5 min at 25°C in import buffer supplemented with NADH (1 mM). After the initial incubation, reaction 3, which had the 4-fold volume compared with reactions 1 and 2, was split into four equal aliquots. One sample was left on ice, the others received either 2 mM ATP (lane 4), 2.5 μM CCCP (lane 5) or 20 μM oligomycin and 40 U/ml apyrase (lane 6). Samples were incubated further for 10 min and then processed as described in (A).

Steps in the ΔΨ- and mtHsp70/MIM44-dependent reaction cycle that initiate protein import into the matrix were examined herein. Translocation intermediates which span both mitochondrial membranes were used to define discrete reactions that occur to transfer the presequence across the inner membrane. A ΔΨ requirement for import was demonstrated after the presequence had entered the matrix and could form a complex with mtHsp70. This finding was significant because it suggests that the presequence can oscillate in the inner membrane during passage to the matrix. Furthermore, a new protein translocation intermediate has been defined. This import intermediate is a membrane-spanning polypeptide that is in a complex with mtHsp70, but is free to diffuse in the import channel. It is generated when the mitochondrial matrix is loaded with the non-hydrolyzable ATP analog adenylyl imidodiphosphate (AMP-PNP). The ATP-form of mtHsp70 appears to initiate interactions between the mtHsp70/MIM44 complex and incoming chains. These data are consistent with a model in which ΔΨ and mtHsp70/MIM44 cooperate to drive initial stages of import via a mechanism that traps incoming chains in the matrix.

Results

A ΔΨ requirement for completion of protein import after the presequence has entered the matrix

Import of the fusion protein pSu9(1–86)–DHFR, produced by fusing a portion of the N-terminal region of *Neurospora crassa* F_o-ATPase subunit 9 precursor (pSu9) in frame to murine dihydrofolate reductase (DHFR) (Ungermann *et al.*, 1994), was arrested by methotrexate (Mtx) after its presequence was translocated across the inner membrane. At this stage, the p-form accumulated with mitochondria, and processing of the presequence was very inefficient

(Figure 1A). In this situation, a stretch of ~35 amino acid residues of the 66 amino acid Su9 presequence has entered the matrix (Ungermann *et al.*, 1994). The majority of the Mtx-arrested pSu9(1–86)–DHFR was held in close apposition to the outer membrane as it was largely resistant to digestion by proteinase K (Schwarz *et al.*, 1993). The arrested pSu9(1–86)–DHFR could be cross-linked to mtHsp70, MIM17, MIM23 and MIM44, suggesting that it is present in the putative MIM import channel (Figure 1B).

To further define the import steps that require ΔΨ, Mtx-liganded pSu9(1–86)–DHFR was accumulated with mitochondria in an initial reaction. Further incubation in the absence of Mtx resulted in the efficient translocation of pSu9(1–86)–DHFR into the matrix (Figure 1A, lane 4). Addition of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) inhibited further import of membrane-spanning pSu9(1–86)–DHFR by >90% (Figure 1A, lane 4 versus 5). Depletion of matrix ATP blocked further import of this same import intermediate by >70% in the second incubation (Figure 1A, lane 4 versus 6). ΔΨ could be required to drive the import of that portion of the Su9 presequence that had not been translocated across the inner membrane. This was ruled unlikely because amino acid residues 36–66 of the presequence contain a low positive charge density and are very inefficient at directing the import of DHFR into mitochondria. Indeed, a fusion protein pSu9(35–94)–DHFR, lacking the first 35 amino acid residues, was imported with an efficiency of <1% as compared with pSu9(1–94)–DHFR or pSu9(1–48)–DHFR. Furthermore, a construct where the second part of the pSu9 presequence was replaced by a mature sequence from cytochrome b_2, pSu9(1–45)+41–DHFR, behaved like pSu9(1–86)–DHFR in our experiments (data not shown). CCCP addition did not inhibit import through interference with ATP metabolism or mtHsp70 function; mitochondrial protein synthesis and mtHsp70-dependent degradation of misfolded matrix proteins (Hermann *et al.*, 1994; Wagner *et al.*, 1994) were not diminished under these conditions (data not shown). Thus, after the presequence has entered the matrix far enough to form a complex with mtHsp70 and MIM44, ΔΨ is still required to facilitate the import process.

What role does ΔΨ play in import after the presequence has entered the matrix? The selective dissipation of ΔΨ with CCCP or depletion of matrix ATP with oligomycin and apyrase to inactivate mtHsp70 (Cyr *et al.*, 1993; Stuart *et al.*, 1994b) both caused Mtx-arrested pSu9(1–86)–DHFR to slide backwards in the import channel. Mtx-arrested pSu9(1–86)–DHFR became sensitive to digestion by proteinase K and was released from mitochondria into the supernatants of reaction mixtures under these conditions (Figure 1C). Thus, ΔΨ and mtHsp70 cooperate during early stages of import to stabilize the initial segments of incoming polypeptides in the mitochondrial matrix. The presequence appears to oscillate in the MIM import channel during import, and ΔΨ is required to facilitate repeated insertion/translocation across the inner membranes.

MtHsp70 binds mature regions of incoming chains with higher affinity than the presequence

The energy requirements for stabilizing import intermediates exposing ~35 amino acid residues of the presequence as compared with the length of mature polypeptide were determined. To generate an import intermediate with the latter characteristics, a 66 amino acid linker was inserted between the pSu9 targeting signal and DHFR domain of pSu9(1–86)–DHFR to form pSu9(1–86)+66–DHFR. pSu9(1–86)+66–DHFR was imported efficiently into mitochondria. It accumulated as a membrane-spanning import intermediate that was processed twice to the mature (m)-form in the presence of Mtx (Figure 2A). Almost all of Mtx-arrested mSu9(1–86)+66–DHFR associated with mitochondria was resistant to digestion by protease. In contrast, only ~50% of the arrested pSu9(1–86)–DHFR was resistant to protease digestion (Figure 1C versus Figure 2A).

The energy requirements for stable association of the import intermediates of mSu9(1–86)+66–DHFR and pSu9(1–86)–DHFR with mitochondria were analyzed (Figure 2B). After accumulation, the Mtx-arrested forms of mSu9(1–86)+66–DHFR and pSu9(1–86)–DHFR were slowly released from fully energized mitochondria. It took ~10 min to release 80% of the membrane-spanning pSu9(1–86)–DHFR from the import apparatus in ATP-depleted mitochondria, but 30 min to release the same amount of mSu9(1–86)+66–DHFR. Furthermore, dissipation of ΔΨ did not promote the release of mSu9(1–86)+66–DHFR, but strongly promoted release of pSu9(1–86)–DHFR. In addition, a ΔΨ requirement for further import of arrested mSu9(1–86)+66 was not observed (data not shown).

When matrix ATP was held at high levels, ~5-fold more mSu9(1–86)+66–DHFR could be co-immunoprecipitated with mtHsp70 than pSu9(1–86)–DHFR (Figure 2C). When matrix ATP was depleted, the level of mtHsp70 binding to mSu9(1–86)+66–DHFR was much higher than to pSu9(1–86)–DHFR. This higher relative affinity of mtHsp70 for the portions of mSu9(1–86)+66–DHFR exposed in the matrix strongly correlated with its stability in mitochondrial membranes.

In summary, mtHsp70 has a higher affinity for the mature regions of the precursor protein investigated than for the presequence. This may allow the mtHsp70/MIM44 import machinery to hold processed incoming chains tightly in the import channel. During import of the presequence on the other hand, interaction of the precursor with the mtHsp70/MIM44 complex appears to be of low affinity. The presequence can slip backwards out of the import channel and the continuous presence of ΔΨ is necessary in this situation.

Two different forms of mtHsp70 can interact with protein translocation intermediates

Once bound to the mtHsp70/MIM44 complex, how is the incoming chain transferred deeper into the matrix? It was proposed that, after ATP hydrolysis, a complex between mtHsp70 and the incoming chain dissociates from MIM44 (Schneider *et al.*, 1994). This would allow the mtHsp70/preprotein complex to move deeper into the matrix with the binding of a further mtHsp70 to the incoming chain via MIM44 (Schneider *et al.*, 1994).

A testable prediction of this model is that two different forms of mtHsp70 bind to segments of the same incoming chain, one in conjunction with MIM44 and the other independently of MIM44. Complexes between MIM44,

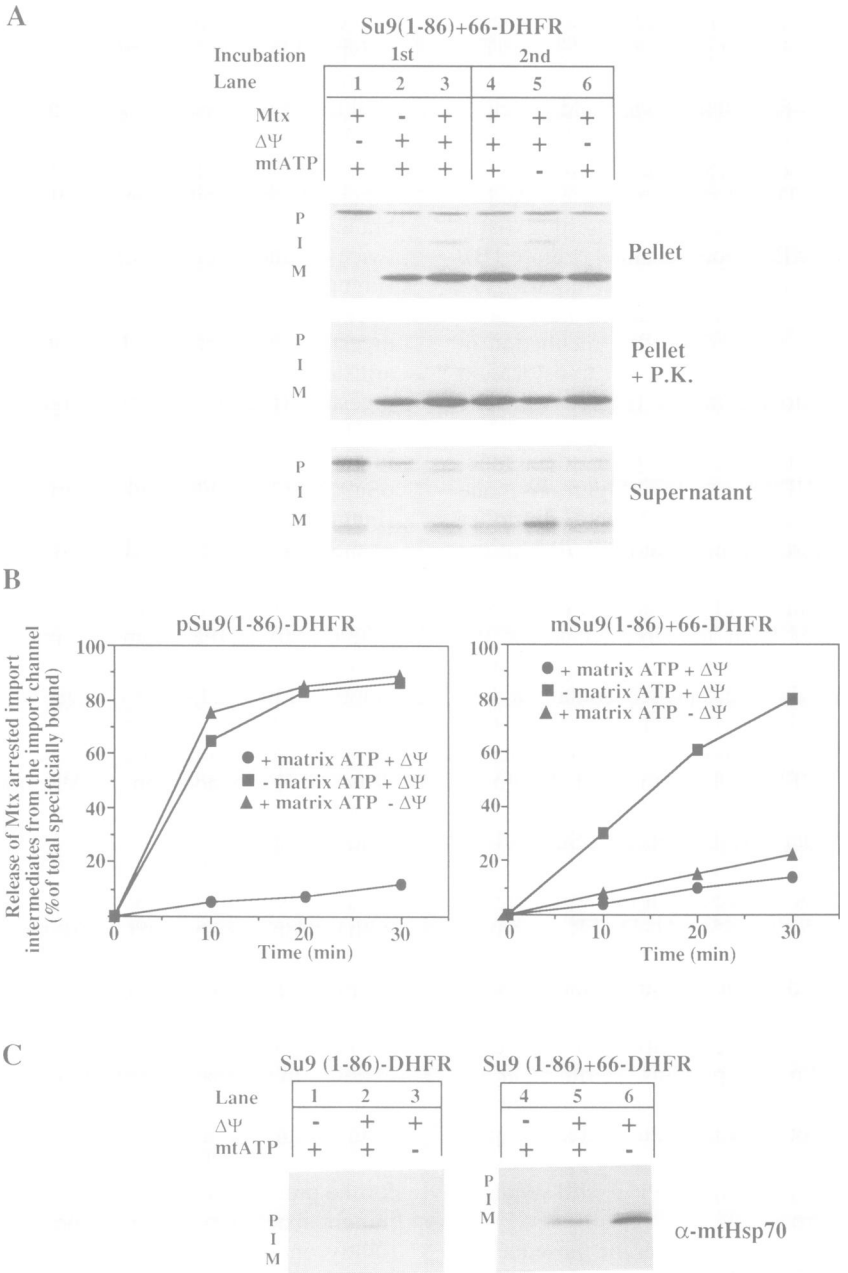

Fig. 2. An Mtx-arrested DHFR fusion protein that extends a non-presequence motif across the inner membrane is stably associated with mitochondria in the absence of ΔΨ. (**A**) Mtx-arrested mSu9(1–86)+66–DHFR is released from the import channel by depletion of ATP, but not by dissipation of ΔΨ. ^{35}S-Labeled pSu9(1–86)+66–DHFR was incubated with mitochondria (250 μg/ml) for 5 min at 25°C. Sample 3, which had the 4-fold reaction volume, was split into four equal aliquots. One sample was kept on ice (lane 3), the others were supplemented with either 2 mM ATP (lane 4), 20 μM oligomycin and 40 U/ml apyrase (lane 5) or 2.5 μM CCCP and 2 mM ATP (lane 6). Incubation was continued for 10 min at 25°C. Samples were processed and analyzed as in Figure 1. (**B**) Release kinetics of Mtx-arrested import intermediates from the translocation channel. ^{35}S-Labeled pSu9(1–86)–DHFR and pSu9(1–86)+66–DHFR were incubated in a 1 ml reaction volume with mitochondria for 5 min at 25°C in the presence of Mtx. Each sample (1 ml) was split into three equal reactions. +matrix ATP +ΔΨ denotes that incubations contained 2 mM ATP, –matrix ATP +ΔΨ denotes addition of 2 mM ATP and 2.5 μM CCCP, and +matrix ATP –ΔΨ denotes addition of 40 U/ml apyrase and 20 μM oligomycin. At the indicated time points, 100 μl of the respective reaction were removed. Acetone precipitates of precursor proteins in the mitochondrial supernatant fraction were analyzed by SDS–PAGE and fluorography. Precursor Su9(1–86)–DHFR and mature Su9(1–86)+66–DHFR size bands were quantified by laser densitometry. (**C**) MtHsp70 binds non-presequence membrane-spanning segments with higher affinity. ^{35}S-Labeled pSu9(1–86)–DHFR and pSu9(1–86)+66–DHFR were incubated with mitochondria for 5 min at 25°C in the presence of Mtx. Co-immunoprecipitation against mtHsp70 was carried out as described in Materials and methods. –ΔΨ denotes the addition of 1 μM valinomycin to the reaction mix.

mtHsp70 and incoming chains are disrupted when matrix ATP is depleted (Schneider *et al.*, 1994; Ungermann *et al.*, 1994). In contrast, ATP depletion stabilizes the interaction of free mtHsp70 with incoming chains that have reached a stage of import beyond the mtHsp70/MIM44 complex (Kang *et al.*, 1990).

To observe such interactions, pSu9(1–86)–DHFR was imported into mitoplasts. Approximately 25 more amino acid residues of a Mtx-arrested precursor are translocated into mitoplasts as compared with mitochondria, because import occurs across a single membrane (Glick *et al.*, 1993; Stuart *et al.*, 1994b). In the presence of Mtx, pSu9(1–

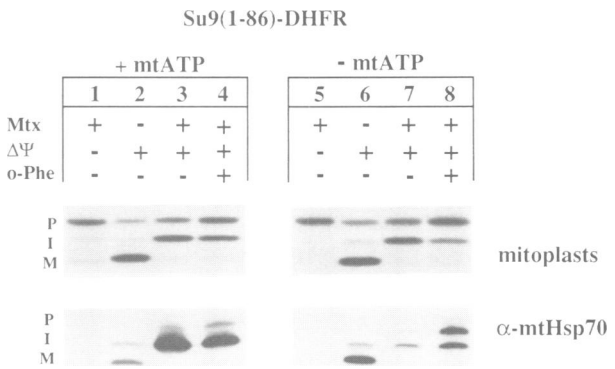

Fig. 3. Two different forms of mtHsp70 are associated with a membrane-spanning translocation intermediate. Import of pSu9(1–86)–DHFR into mitoplasts. The MOM was disrupted to generate mitoplasts by incubation of isolated mitochondria in 20 mM HEPES, pH 7.4, for 30 min on ice. Mitoplasts were reisolated by centrifugation and resuspended in 250 mM sorbitol, 1 mM EDTA and 10 mM MOPS, pH 7.2. ^{35}S-Labeled pSu9(1–86)–DHFR was incubated with mitoplasts (250 μg/ml) in 200 μl of import buffer at 25°C for 5 min in the presence or absence of Mtx. *o*-Phenanthroline (*o*-phe; 0.8 mM) and EDTA (12 mM) were added to inhibit the activity of the MPP. In the presence of *o*-phe and EDTA, no defects in protein translocation into the matrix space were observed (data not shown), suggesting that these chelators act specifically on MPP. ΔΨ was dissipated by addition of valinomycin (1 μM). After the initial import reaction, each sample was split. Samples analyzed in lanes 5–8 were incubated for a further 5 min at 25°C. Prior to this incubation, lane 5 was mock-treated and lanes 6–8 received 20 μM oligomycin and 40 U/ml apyrase to deplete the matrix of ATP. After the second incubation, samples were placed on ice. A 20 μl aliquot was then removed from all samples and used for the analysis of protein import and protease sensitivity of import intermediates as previously described ('mitoplasts'). The remainder of each sample (80 μl) was then used for analysis of complexes that formed between mtHsp70 and Su9(1–86)–DHFR in co-immunoprecipitation assays, as described in Materials and methods ('α-mtHsp70').

86)–DHFR was imported to a position in mitoplasts where its presequence could now be processed efficiently to the intermediate (i)-form. After processing, ~15 amino acid residues of the Mtx-arrested i-form of Su9(1–86)–DHFR are present in the matrix of mitoplasts (Ungermann *et al.*, 1994). This import intermediate was co-immunoprecipitated efficiently with mtHsp70 when matrix ATP levels were high. Upon lowering the levels of matrix ATP, the quantity of iSu9(1–86)–DHFR that could be co-immunoprecipitated with mtHsp70 was reduced by >90% (Figure 3, lane 3 versus 7). This result is consistent with the retrograde diffusion of import intermediates out of the import channels under these conditions (see Figure 1C). Similar to the findings in experiments with intact mitochondria (Figure 1C), import intermediates that remained associated with ATP-depleted mitoplasts were completely sensitive to digestion by proteinase K (data not shown). The i-form of Su9(1–86)–DHFR is therefore stabilized in the inner membrane through its association with the Hsp70/MIM44 complex.

The length of imported chain exposed in the matrix of mitoplasts was extended to ~50 amino acid residues by blocking cleavage of the first 35 amino acid residues of the presequence with *o*-phenanthroline (*o*-phe) (Hartl *et al.*, 1989). The concentration of *o*-phe used in these experiments was set so that processing of pSu9(1–86)–DHFR by the mitochondrial processing peptidase (MPP) was inhibited by ~30%. Under these conditions both the p- and i-forms of Su9(1–86)–DHFR accumulated with energized mitoplasts and could be co-immunoprecipitated in similar quantities with mtHsp70 (Figure 3, lane 4). When matrix ATP was depleted, the p-form was stably associated with mitoplasts in contrast to the i-form (Figure 3, lane 4 versus 8). Release of intermediate-sized Su9(1–86)–DHFR from the import channel correlated with the decreased complex formation with mtHsp70 at low matrix ATP (Figure 3, lane 4). In contrast, the Mtx-arrested precursor form of Su9(1–86)–DHFR that could be co-immunoprecipitated with mtHsp70, increased several-fold upon matrix ATP depletion.

Thus, both the MIM44-bound and the free forms of mtHsp70 can be detected in a complex with an import intermediate arrested at a very early stage of import. Since the quantity of the mtHsp70 bound to pSu9(1–86)–DHFR was several-fold higher when ATP levels were low, it appears that interaction between the free form of mtHsp70 and the precursor is tighter than that between incoming chains and the mtHsp70/MIM44 complex.

Interference with the mtHsp70/MIM44 cycle allows incoming chains to slide back and forth in the import channel

To address the mechanism by which polypeptides are held tightly in the import machinery, the activity of mtHsp70 was reversibly inhibited with the non-hydrolyzable ATP analog AMP-PNP (Liberek *et al.*, 1992) and the import and processing of pSu9(1–94)–DHFR precursor proteins was examined (Figure 4). pSu9(1–94)–DHFR was used in these experiments because the Mtx-arrested form of this precursor is efficiently processed to the i-form. When mitochondria were pretreated for 10 min with ATP or AMP-PNP, pSu9(1–94)–DHFR was efficiently translocated to the matrix and processed (Figure 4A). If mitochondria were first depleted of matrix ATP and then incubated with AMP-PNP no import was observed (data not shown). This suggests that mere binding of an ATP analog to mtHsp70 is insufficient to drive import.

On the other hand, pretreatment of mitochondria with AMP-PNP led to almost complete inhibition of the processing of Mtx-arrested pSu9(1–94)–DHFR to the i-form, and the p-form accumulated with mitochondria (Figure 4B). This effect of AMP-PNP on presequence processing was specific because the p-form of Su9(1–94)–DHFR that accumulated with mitochondria under these conditions could be converted rapidly to the i-form and dissociate from mitochondria if ATP was added in excess of AMP-PNP (Figure 4C). Upon prolonged incubation in the presence of AMP-PNP, the Mtx-arrested p-form of Su9(1–94)–DHFR was processed to the i-form (Figure 4D). The rate at which iSu9(1–94)–DHFR was released from mitochondria was not affected by AMP-PNP (Figure 4D). Thus, pretreatment of mitochondria with AMP-PNP caused a kinetic defect in the processing. Fully imported precursor proteins were processed efficiently in AMP-PNP-treated mitochondria, thus the processing defect observed for Mtx-arrested intermediates is due to their interaction with the import machinery and not a result of inactivation of MPP.

In energized mitochondria, the mtHsp70/MIM44 complex holds Mtx-arrested import intermediates in close apposition to the outer membrane and they are largely

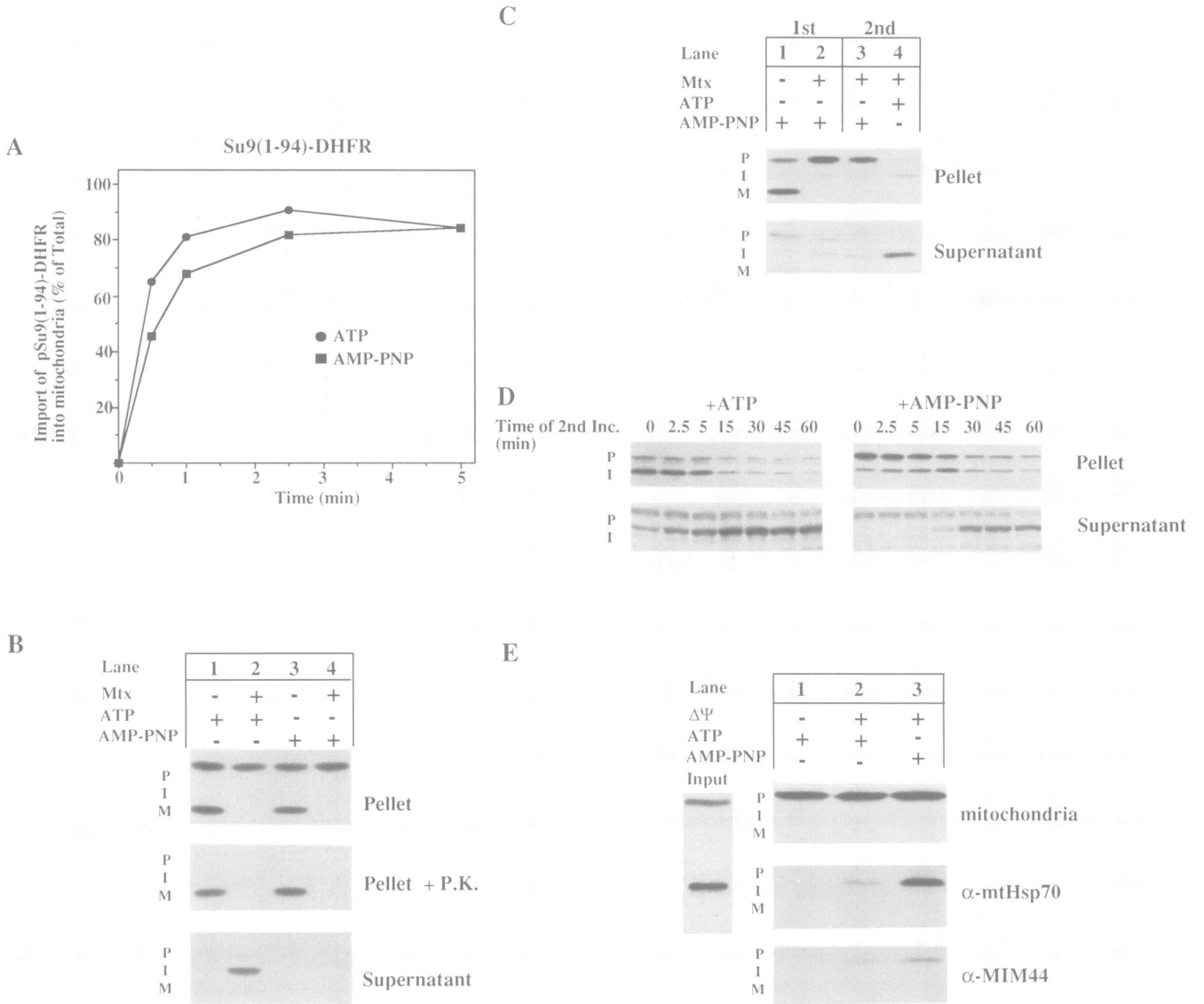

Fig. 4. Interference with the MIM44/Hsp70-driven cycle by addition of the non-hydrolyzable analog AMP-PNP causes inefficient import and processing of arrested precursor proteins. (**A**) Addition of AMP-PNP to mitochondria results in a delay of import into the matrix. +AMP-PNP and +ATP denote that mitochondria (250 μg/ml) were pretreated in a 500 μl reaction volume with either 10 mM AMP-PNP or 10 mM ATP (for details see Materials and methods). ^{35}S-Labeled pSu9(1–94)–DHFR was incubated at 25°C with mitochondria. At the indicated time points, 100 μl samples of the respective reaction were removed and analyzed for protease resistance. Analysis of protected material was done by SDS–PAGE and fluorography. Protected mature sized bands were quantified by laser densitometry. (**B**) Mtx-arrested pSu9(1–94)–DHFR does not get processed in the presence of AMP-PNP and accumulates with mitochondria. ^{35}S-Labeled pSu9(1–94)–DHFR was imported into mitochondria preincubated with ATP or AMP-PNP for 2.5 min at 25°C. Precursor proteins in the mitochondrial pellet, supernatant and protease-resistant fraction were analyzed by SDS–PAGE and fluorography. (**C**) Chase of unprocessed pSu9(1–94)–DHFR to the i-form by addition of ATP. Two successive incubations were carried out. ^{35}S-Labeled Su9(1–94)–DHFR was incubated with AMP-PNP-pretreated mitochondria for 2.5 min at 25°C. Reaction 2, which had the 3-fold volume compared with lane 1, was divided into three equal aliquots. One was kept at 0°C (lane 2), the other two were reisolated by centrifugation and resuspended in import buffer containing either 10 mM AMP-PNP (lane 3) or 10 mM ATP (lane 4). Incubation was continued for 10 min at 25°C. Samples were analyzed for mitochondrial pellet and supernantant fraction. (**D**) AMP-PNP causes kinetic delay of processing of Mtx-arrested pSu9(1–94)–DHFR. ^{35}S-Labeled pSu9(1–94)–DHFR was incubated in a 700 μl reaction volume with ATP- or AMP-PNP-pretreated mitochondria at 25°C. At the indicated time points, 100 μl samples were removed and processed for mitochondrial pellet and supernatant fraction. Samples were analyzed by SDS–PAGE and fluorography. (**E**) MtHsp70, but not MIM44, is tighly associated with the translocation intermediate. ^{35}S-Labeled Su9(1–94)–DHFR was imported in a 200 μl reaction volume into pretreated mitochondria for 2.5 min at 25°C in the presence of Mtx. EDTA (12 mM) and *o*-phe (0.8 mM) were added to inhibit processing by MPP. After the initial import reaction, samples were split into three aliquots. One was analyzed for import (mitochondria), the other two were reisolated, and co-immunoprecipitation with antiserum against mtHsp70 (α-mtHsp70) and MIM44 (α-MIM44) was carried out as described in Materials and methods. Input represents 25% (mitochondria) and 3% of lysate (α-mtHsp70, α-MIM44) added to the reaction mixture, respectively.

resistant to digestion by proteinase K (see Figures 1C and 2A). The Mtx-arrested pSu9(1–94)–DHFR that accumulated with AMP-PNP-treated mitochondria became completely digested by added proteinase K (Figure 4B). AMP-PNP also caused Mtx-arrested pSu9(1–86)–DHFR, initially accumulating in the presence of matrix ATP, to slide back in the import channel and become sensitive to protease digestion (data not shown). AMP-PNP apparently interferes with the ability of the MtHsp70/MIM44 complex to stabilize polypeptides in the import machinery.

A fraction of the p-form of Su9(1–94)–DHFR could be co-immunoprecipitated specifically with both mtHsp70 and MIM44 when arrested across the membranes of energized mitochondria by Mtx (Figure 4E). The quantity of the complex between MIM44 and the incoming chain was only 10% of that observed with mtHsp70. Pretreatment of mitochondria with AMP-PNP resulted in a large increase in the quantity of Mtx-arrested pSu9(1–94)–DHFR that could be co-immunoprecipitated with mtHsp70, but not with MIM44. Thus, the complex between mtHsp70 and the presequence has a longer half-life in the presence of AMP-PNP. The stabilization of complexes between Hsp70 proteins and substrates by AMP-PNP may also contribute to the observed defects in the processing of Mtx-arrested import intermediates.

What is the explanation for enhanced complex formation between the incoming chain and mtHsp70 by AMP-PNP, and the reduced fixing of the polypeptides in the import channel? After binding the incoming chain, the ternary complex between the mtHsp70, MIM44 and the incoming chain may dissociate rapidly. Indeed, the quantities of import intermediate detected in a complex with either mtHsp70 or MIM44 are not identical (Figure 4E). After binding the incoming chain, mtHsp70 appears to dissociate from MIM44. AMP-PNP stabilizes the resulting complex between mtHsp70 and translocation intermediates. For steric reasons, the stabilization of the complex between the free form of mtHsp70 and the incoming chain would then reduce the rate at which the MIM44-bound form of mtHsp70 can rebind the incoming chain. As a consequence, the complex between the AMP-PNP form of mtHsp70 and the Mtx-arrested import intermediate would be able to oscillate in the import channel because it is not fixed efficiently to the import machinery by MIM44.

Discussion

The data presented here can be summarized in a model of the reaction sequence that occurs to drive the initial steps in protein translocation into the mitochondrial matrix (Figure 5). Translocation of the presequence across the inner membrane is initiated by the ΔΨ-dependent import step (Schleyer and Neupert, 1985). ΔΨ is not sufficient to hold the presequence in the MIM import channel. Interaction of the presequence with the mtHsp70/MIM44 complex is required to trap it in the matrix (Cyr *et al.*, 1993; Ungermann *et al.*, 1994). ATP hydrolysis stabilizes the complex between mtHsp70 and the incoming chain. The mtHsp70/MIM44 complex binds the presequence with low affinity compared with mature portions of precursor protein. This allows the presequence to slide in and out of the MIM import channel during passage to the matrix. The ΔΨ- and ATP-dependent import components must cooperate at this import step to make presequence translocation efficient. The ternary complex between mtHsp70, MIM44 and the incoming chain does not appear to be stable. MtHsp70 and the precursor protein dissociate from MIM44 as a complex during or after ATP hydrolysis. This unlocks the incoming chain from MIM44. The precursor protein which remains bound to the free form of mtHsp70 can diffuse in both directions in the import channel. Diffusion out of the channel is limited by the presence of mtHsp70 on the incoming polypeptide chain. When

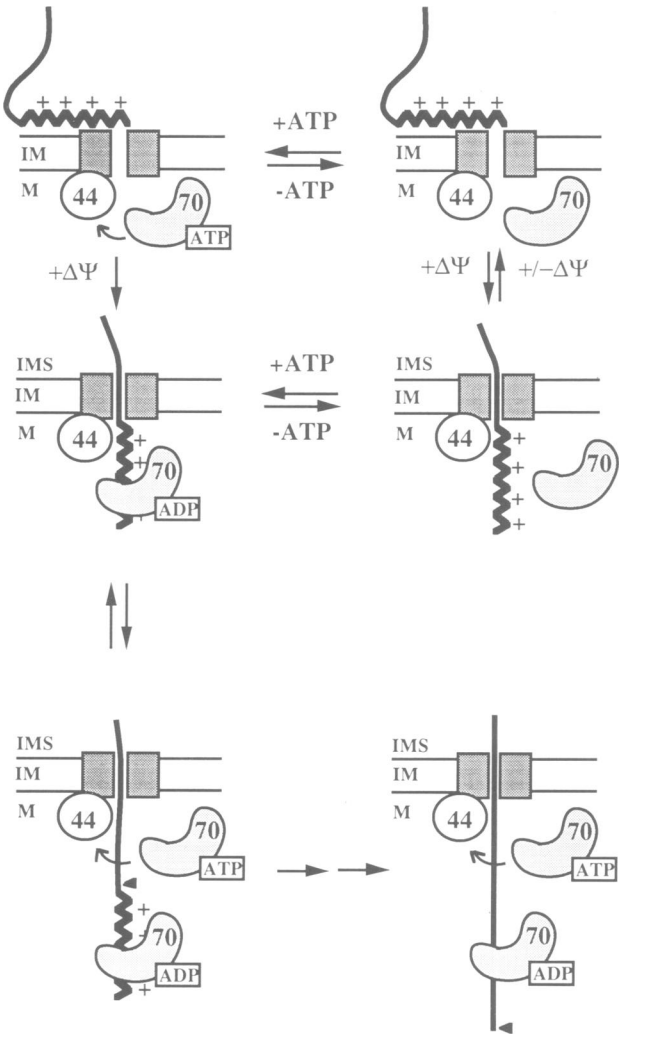

Fig. 5. A model for the role of ΔΨ, matrix ATP and the mitochondrial proteins MIM44 and Hsp70 in presequence translocation across the inner mitochondrial membrane. Zig-zag lines and positive charges on the precursor protein indicate the presequence. The arrowhead shows the MPP cleavage site. Other abbreviations are as follows: IMS, intermembrane space; IM, inner membrane; M, matrix; 44, MIM44; 70, mtHsp70. Rectangles denote the inner membrane protein translocation apparatus. C-terminal portions of the preprotein are not drawn.

diffusion of the precursor protein into the matrix is deep enough, the binding of a second mtHsp70 to the precursor protein via MIM44 locks the chain in the import channel. This serves to prevent retrograde movement of proteins in the import channel and allows for net movement of polypeptide into the matrix. MGE, a mitochondrial homolog of the bacterial GrpE nucleotide release factor, appears to mediate the dissociation of the mtHsp70 from the incoming chain (Bolliger *et al.*, 1994; Laloyara *et al.*, 1994; Pfanner *et al.*, 1994; Westermann *et al.*, 1995). Repeated cycles of MIM44-mediated binding and release of mtHsp70 to the incoming chain drive the completion of the import process (Schneider *et al.*, 1994).

A number of steps in this working model require further discussion. A first point is the suggestion that the ATP-form of mtHsp70 is required to initiate its interaction with the incoming chain. This interpretation is supported by the observation that increasing the ADP/ATP ratio in the

mitochondrial matrix causes the release of membrane-spanning intermediates from mitochondria. The ADP-form of mtHsp70 does not appear to bind the incoming chain with fast enough kinetics to stabilize polypeptides in the import channel. Consistently, the ATP-form of purified Hsp70 has a higher on-rate for substrate binding than the ADP-form (Schmid *et al.*, 1994). ATP hydrolysis is required to stabilize complexes between Hsp70 and substrate proteins because the ADP-form has a higher affinity for substrate protein (Palleros *et al.*, 1991, 1993).

Thermal energy has been proposed to drive the movement of polypeptides across mitochondrial membranes (molecular ratchet model) (Neupert *et al.*, 1990). In this model, mtHsp70 serves to trap segments of polypeptides in the matrix and confer unidirectionality on the import process. On the other hand, the possibility has been discussed that conformational changes in mtHsp70 occurring upon hydrolysis of ATP are sufficient to assist the diffusion of proteins across the membrane by 'pulling' them into the matrix (Glick, 1995; Pfanner and Meijer, 1995). The data presented here demonstrate that polypeptides can slide in the MOM and MIM import channels independently of energy input.

One aspect of the molecular ratchet model is that mtHsp70 remains bound to the incoming chain after it has hydrolyzed ATP and is released from MIM44. Two observations we present herein support this interpretation. First, in spite of the fact that MIM44 and mtHsp70 are found in a 1:1 complex in the absence of import intermediate (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994), significantly more mtHsp70 than MIM44 was found in a complex with membrane-spanning import intermediates. Second, when mitochondria were treated with AMP-PNP, a large increase in the quantity of import intermediate that can be co-immunoprecipitated with mtHsp70, but not with MIM44, was observed. Under these same conditions, polypeptides that are normally locked tightly in the import channel were free to oscillate in the mitochondrial membranes. The translocation intermediate of pSu9(1–94)–DHFR used in these experiments was arrested at a stage of import where only the MIM44-bound form of mtHsp70 can gain access to the incoming chain (Ungermann *et al.*, 1994). Therefore, a new import intermediate that was predicted by the molecular ratchet model has been identified. It is inserted across the inner membrane and in complex with mtHsp70, but not locked tightly in the import channel.

The reversible nature of these early steps suggests that it would be advantageous for proteolytic removal of the presequence to occur at a later stage of import. In support of this, we find that at least one round of mtHsp70 binding and release from the incoming chain occurs prior to the MPP processing event. If release of mtHsp70 from the presequence is prevented, then MPP processing is inhibited. MtHsp70 may sterically hinder access of MPP to the presequence when it is in close proximity to the inner membrane import machinery. In this way, processing of the presequence seems to be prevented until sufficient polypeptide has entered the matrix to ensure the unidirectionality of the process.

The mechanisms which determine the specificity of the interactions between Hsp70 family members and substrate proteins are a topic of considerable interest (Gething and Sambrook, 1992). A general consensus is emerging that Hsp70 proteins prefer to bind polypeptides that are in extended conformations and are enriched in hydrophobic amino acid residues (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993). As demonstrated here, mtHsp70 binds regions of incoming chains with varying affinities. Since the presequence is enriched in polar and charged amino acid residues, it is not surprising that its interaction with mtHsp70 is of low affinity when compared with mature regions. A potential role for MIM44 in the import process might be to help preserve the extended conformation of polypeptides as they arrive in the matrix and to present them to mtHsp70 in a preferred conformation. This would be analogous to the role of DnaJ-like proteins which assist Hsp70 homologs in mediating cellular protein metabolism (Langer *et al.*, 1992; Georgopoulos and Welch, 1993; Silver and Way, 1993; Cyr *et al.*, 1994).

The differences in the affinity of mtHsp70 for regions of incoming chains imparts inefficiency to the import process. Through the rapid recycling of mtHsp70 onto the incoming precursor chain, MIM44 may help to overcome these problems.

Materials and methods

Protein translocation into isolated yeast mitochondria

Mitochondria were isolated from yeast strain D27310-B grown in a semi-synthetic lactate medium according to published procedures (Daum *et al.*, 1982). Isolated mitochondria were suspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) at 10 mg protein/ml, snap-frozen in liquid nitrogen and stored at –80°C. Just prior to use in import reactions, an aliquot of mitochondria was thawed and placed on ice. Mitochondria (250 μg/ml) were incubated in import buffer [500 mM sorbitol, 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 80 mM KCl, 2 mM potassium phosphate and 0.01% fatty acid-free bovine serum albumin (BSA)]. Energy sources such as ATP and NADH were added as indicated in the figure legends. Import reactions were started by addition of ^{35}S-labeled precursor protein synthesized by *in vitro* transcription and translation in rabbit reticulocyte lysate (Söllner *et al.*, 1991; Cyr *et al.*, 1995). Incubations were carried out at 25°C for the times indicated, and import reactions were stopped on ice. Mitochondria were reisolated at 12 000 *g* for 10 min in a refrigerated centrifuge at 4°C. The mitochondrial pellets were analyzed by electrophoresis on 12% SDS–polyacrylamide gels, followed by fluorography of the dried gels to detect radiolabeled proteins. The mobility shift of precursor proteins on SDS–PAGE gels after $\Delta\Psi$-dependent processing of the presequence by the MPP provided a convenient assay for protein translocation into mitochondria (Hartl *et al.*, 1989).

Construction of pSu9–DHFR fusion proteins

N-terminal regions of various lengths of the *N.crassa* pSu9 (Viebrock *et al.*, 1982) were fused in-frame to murine DHFR by PCR using standard techniques. The pSu9 presequence contains two sites for MPP processing at position 35 (i-form) and at position 66 (m-form). pSu9(1–86)+66–DHFR was constructed by inserting amino acids 210–276 of mature *Saccharomyces cerevisiae* cytochrome b_2 into a *Bam*HI site between the presequence and the DHFR coding sequence of pGEM4-pSu9(1–86)–DHFR (Ungermann *et al.*, 1994).

Cross-linking of import intermediates to components of the import machinery

^{35}S-Labeled pSu9–DHFR (10–20 μl) was incubated for 5 min at 25°C in a 200 μl reaction mixture containing isolated mitochondria (250 μg/ml) and then placed on ice. The reaction mixture was made 200 μM in the lysine-specific cross-linker disuccinimidyl suberate (DSS; Pierce) dissolved fresh in dimethylsulfoxide (DMSO), and incubated for an additional 30 min on ice (Söllner *et al.*, 1992). The cross-linking reaction was then quenched by addition of Tris–HCl, pH 7.4, to 100 mM and then split. Mitochondria from one half of the reaction mixture were immediately reisolated, and cross-linked products were determined by

monitoring the shift in the mobility of ^{35}S-labeled pSu9–DHFR upon electrophoresis on SDS–polyacrylamide gels (Söllner *et al.*, 1992).

To determine which components of the import apparatus formed a cross-linked adduct with Mtx-arrested pSu9–DHFR, mitochondria from the other half of the reaction were reisolated, resuspended in 40 µl of lysis buffer A [1.0% Triton, 5 mM EDTA, 10 mM Tris–HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 300 mM NaCl and 1% BSA] supplemented with 1% SDS and incubated on ice for 15 min. Solubilized mitochondria were diluted to 1.0 ml with buffer A. Insoluble material was removed from the reaction mixtures by centrifugation at 15 000 *g* for 10 min at 4°C. MIM44, MIM23 or MIM17 coupled to protein A–Sepharose beads were added to the supernatant antibodies against Hsp70. After 1 h of incubation at 4°C on a shaker, beads were isolated by centrifugation and washed twice with buffer A without BSA. Finally, proteins were released from beads by addition of SDS sample buffer and boiling. Cross-linked products were analyzed by SDS–PAGE.

Co-immunoprecipitation of protein translocation intermediates with the MIM import machinery

^{35}S-Labeled precursor protein was incubated with isolated mitochondria in the presence of Mtx (3 µM) to allow import intermediates to accumulate with mitochondria as described previously (Rassow *et al.*, 1992). Mitochondria were reisolated by centrifugation and the pellet was solubilized in 150 µl of lysis buffer (0.1% Triton X-100, 110 mM NaCl, 5 mM EDTA, 10 mM MOPS, pH 7.2, 1 mM PMSF and 0.5% BSA). After 10 min of incubation on ice, an additional 350 µl of lysis buffer was added. Insoluble material was removed by centrifugation at 15 000 *g* for 15 min. Protein A–Sepharose-coupled antibodies to mtHsp70 or MIM44 were added to the supernatant and the reaction was incubated as before. The protein A–Sepharose beads were pelleted by centrifugation and washed twice with 500 µl of lysis buffer.

SDS sample buffer without β-mercaptoethanol was added to the beads and samples were boiled to release bound proteins. Radiolabeled proteins isolated by this procedure were analyzed by SDS–PAGE and fluorography.

Antibodies were coupled to protein A–Sepharose beads by the following protocol. Protein A–Sepharose slurry (30 µl) obtained from the manufacturer (Pharmacia) was mixed with 700 µl of lysis buffer and 30 µl of polyclonal antiserum against mtHsp70 (Wagner *et al.*, 1994). This mixture was incubated for 1 h at 4°C with shaking. The beads were reisolated by centrifugation and used for immunoprecipitation reactions.

Preincubation of mitochondria with AMP-PNP

Mitochondria (250 µg/ml) were diluted into standard reaction buffer and incubated at 25°C for 3 min to reduce preexisting matrix ATP levels. ATP (10 mM) or AMP-PNP (10 mM) were added, and incubation was allowed to proceed for 3 min. NADH (1 mM) was added and, after a further incubation for 3 min, import reactions were started by addition of ^{35}S-labeled pSu9–DHFR.

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