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The nucleotide exchange factor MGE exerts a key function in the ATP-dependent cycle of mt-Hsp70–Tim44 interaction driving mitochondrial protein import

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Import of preproteins into the mitochondrial matrix is driven by the ATP-dependent interaction of mt-Hsp70 with the peripheral inner membrane import protein Tim44 and the preprotein in transit. We show that Mge1p, a co-chaperone of mt-Hsp70, plays a key role in the ATP-dependent import reaction cycle in yeast. Our data suggest a cycle in which the mt-Hsp70–Tim44 complex forms with ATP: Mge1p promotes assembly of the complex in the presence of ATP. Hydrolysis of ATP by mt-Hsp70 occurs in complex with Tim44. Mge1p is then required for the dissociation of the ADP form of mt-Hsp70 from Tim44 after release of inorganic phosphate but before release of ADP. ATP hydrolysis and complex dissociation are accompanied by tight binding of mt-Hsp70 to the preprotein in transit. Subsequently, the release of mt-Hsp70 from the polypeptide chain is triggered by Mge1p which promotes release of ADP from mt-Hsp70. Rebinding of ATP to mt-Hsp70 completes the reaction cycle.

Keywords: Hsp70/mitochondrial protein import/Mge/Tim44

Introduction

Molecular chaperones of the 70 kDa heat shock protein family (Hsp70s) are found in all organisms and in various subcellular compartments (Georgopoulos *et al.*, 1994; Hartl *et al.*, 1994; Morimoto *et al.*, 1994). The general ability of Hsp70s to bind non-native polypeptides in an ATP-dependent manner is utilized to facilitate a variety of different cellular processes. Thus, Hsp70s mediate folding of newly synthesized proteins (Beckmann *et al.*, 1990; Nelson *et al.*, 1992; Frydman *et al.*, 1994), unfolding of folded proteins and their translocation across membranes (Kang *et al.*, 1990; Vogel *et al.*, 1990), stabilization of denatured proteins and protein degradation (Sherman and Goldberg, 1992; Schröder *et al.*, 1993; Wagner *et al.*, 1994), DNA replication (Zylicz *et al.*, 1989; Wickner *et al.*, 1991), regulation of transcription (Gamer *et al.*, 1992) and uncoating of clathrin-coated vesicles (Chappell *et al.*, 1987). Accessory proteins specify the action of Hsp70s by tagging protein substrates and by regulating the ATP-dependent interaction of Hsp70 with these substrates (Liberek *et al.*, 1991; Schneider *et al.*, 1994; Szabo *et al.*, 1994; McCarty *et al.*, 1995).

In mitochondria, Tim44, a peripheral inner membrane protein, and Mge1p, the mitochondrial homologue of bacterial GrpE, cooperate with mt-Hsp70, a major heat shock protein of the mitochondrial matrix, in protein translocation across the inner membrane (Bolliger *et al.*, 1994; Kronidou *et al.*, 1994; Laloraya *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; Voos *et al.*, 1994). Tim44, a component of the translocase of the inner membrane, recruits mt-Hsp70 to the sites of protein import (Berthold *et al.*, 1995). The mt-Hsp70–Tim44 complex interacts with the unfolded preprotein in transit which stimulates the ATP-dependent dissociation of mt-Hsp70 from Tim44 (Schneider *et al.*, 1994). Tim44 and mt-Hsp70 have been proposed to be parts of a molecular ratchet (Schneider *et al.*, 1994) or of a molecular motor (Glick, 1995) which drives unfolding of folded preproteins on the outside of the mitochondria and their translocation across the mitochondrial membranes by multiple cycles of interaction with the translocating polypeptide chain.

Several groups have studied the dependence of the dissociation of the mt-Hsp70–Tim44 complex on nucleotides and nucleotide analogues in order to unravel the various steps of the reaction cycle (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; von Ahsen *et al.*, 1995; Horst *et al.*, 1996). Observed differences of complex stability in mitochondrial lysates and with affinity-purified mt-Hsp70–Tim44 complex have led to different concepts of the reaction cycle. On the one hand it has been proposed that mt-Hsp70 in the ATP form binds to Tim44 and that ATP hydrolysis occurs while mt-Hsp70 is bound to Tim44. Thus, ATP hydrolysis precedes complex dissociation and Hsp70–ADP dissociates from Tim44 (Schneider *et al.*, 1994). On the other hand it was suggested that ATP is hydrolysed by free mt-Hsp70 which then associates with Tim44 (von Ahsen *et al.*, 1995). ADP is released from the mt-Hsp70–Tim44 complex and dissociation of the nucleotide-free complex is triggered by binding of ATP to Hsp70 (Glick, 1995; von Ahsen *et al.*, 1995; Horst *et al.*, 1996).

Mge1p is essential for protein import into the mitochondrial matrix (Laloraya *et al.*, 1994; Westermann *et al.*, 1995). Here we show that Mge1p modulates the nucleotide-dependent stability of the mt-Hsp70–Tim44 complex in the presence of physiological concentrations of cations. Mge1p stabilizes the mt-Hsp70–Tim44 complex in the presence of ATP analogues but not in the presence of hydrolysable ATP, suggesting that the co-chaperone assists in assembly of the ATP form of the complex prior to ATP hydrolysis. Mge1p dissociates the ADP form of the mt-Hsp70–Tim44 complex but not the nucleotide-free form. Thus, Mge1p facilitates complex dissociation prior to release of ADP from mt-Hsp70 and ATP uptake occurs after complex dissociation. Inorganic phosphate has a stabilizing effect on the ADP form of the complex,

indicating that phosphate release precedes complex dissociation triggered by Mge1p. We show that apparent differences in complex stability observed with nucleotide analogues after purification of the mt-Hsp70–Tim44 complex are due to the loss of effective concentrations of Mge1p.

We further show that mt-Hsp70 with the *ssc1-3* mutation (Gambill *et al.*, 1993) has a reduced affinity for both Mge1p and ATP and is defective in nucleotide-dependent interaction with Tim44. Overexpression of Mge1p leads to reduced kinetics of import of folded preproteins, suggesting that Mge1p modulates the release of mt-Hsp70 from preproteins in transit.

Results

Monovalent cations, nucleotides and inorganic phosphate modulate the stability of the mt-Hsp70–Tim44 complex

The stability of the mt-Hsp70–Tim44 complex in response to nucleotides was determined by lysing mitochondria with detergent and measuring the mt-Hsp70 that can be co-precipitated with antibodies against Tim44. Mitochondrial detergent extracts were prepared in the presence of either Na^+ or K^+ ions. These cations affect the rates of binding of ATP to and release from Hsp70 as well as the rate of ATP hydrolysis by Hsp70 (O'Brien and McKay, 1995; Wilbanks and McKay, 1995).

Na^+ ions are likely to exert a stabilizing effect on the mt-Hsp70–Tim44 complex. In fact, in Na^+-containing buffers the mt-Hsp70–Tim44 complex was preserved both in the presence of EDTA and of Mg^{2+} with no added nucleotides (Figure 1A). The mt-Hsp70–Tim44 complex was completely dissociated by ATP and partially by ADP (nucleotides were always added together with Mg^{2+}). The complex was stable in the presence of the ATP analogues ATPγS, AMP-PNP and AMP-PCP. Thus, as reported previously (Schneider *et al.*, 1994), hydrolysable ATP is required to dissociate the mt-Hsp70–Tim44 complex in the presence of Na^+ ions.

The following experiments were performed using buffers containing K^+ ions since the mitochondrial matrix contains high concentrations of K^+ ions (~70 mM) and low concentrations of Na^+ ions (Scarpa, 1979). The mt-Hsp70–Tim44 complex was stable with EDTA (Figure 1A). It dissociated almost completely when Mg^{2+} was added, suggesting that the low concentration of endogenous mitochondrial nucleotides in the mitochondrial lysates was sufficient to destabilize the complex due to the presence of K^+ ions. AMP-PCP efficiently stabilized the mt-Hsp70–Tim44 complex, indicating that this ATP analogue competed with the endogenous nucleotides. The complex dissociated completely in the presence of ATP, and also with ATPγS, AMP-PNP and with ADP. Thus, these nucleotides either facilitated dissociation or were at least unable to compete with endogenous nucleotides (present in ~500-fold lower concentration as compared with intact mitochondria).

Inorganic phosphate (P_i) inhibits the steady state ATPase activity of DnaK, the major bacterial Hsp70 homologue, and, following ATP hydrolysis, release of P_i precedes the release of ADP from DnaK (Banecki and Zylicz, 1996). We asked whether P_i has an influence on the stability of the mt-Hsp70–Tim44 complex. Mitochondria were lysed in the presence or absence of P_i (4 mM). Inorganic phosphate abolished dissociation of the mt-Hsp70–Tim44 complex in mitochondrial lysates with Mg^{2+} alone and also in the presence of ADP (Figure 1B). This suggests that P_i interacts with mt-Hsp70 and stabilizes the mt-Hsp70–Tim44 complex in the ADP+P_i form.

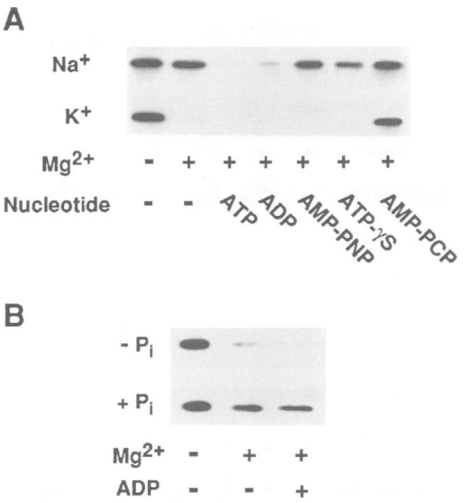

Fig. 1. Nucleotide-dependent dissociation of the mt-Hsp70–Tim44 complex in mitochondrial lysates. Mt-Hsp70 which was co-immunoprecipitated with Tim44 from lysed mitochondria is shown. (**A**) Influence of K^+ and Na^+ ions on the stability of the mt-Hsp70–Tim44 complex. Lysis of mitochondria (200 μg/ml) was carried out in assay buffer (0.5% TX-100, 30 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) containing either 100 mM NaCl (Na^+) or 100 mM KCl (K^+) with the following concentrations of indicated additions: 4.5 mM EDTA (Mg^{2+} –), 5 mM Mg-acetate (Mg^{2+} +), 1 mM ATP, 5 mM ADP, 5 mM AMP-PNP, 5 mM ATPγS or 5 mM AMP-PCP. (**B**) Inorganic phosphate stabilizes the mt-Hsp70–Tim44 complex. Mitochondria were lysed in assay buffer containing 100 mM KCl, 5 mM Mg-acetate and 2 mM ADP in the presence or absence of 4 mM NaH_2PO_4 (P_i). Immunoprecipitations with anti-Tim44 IgGs were performed. Mt-Hsp70 bound to Tim44 was analysed by SDS–PAGE and Western blotting with antibodies specific for mt-Hsp70.

In summary, in mitochondrial lysates the mt-Hsp70–Tim44 complex dissociates with ATP and it is stable with AMP-PCP and ADP+P_i in the presence of both Na^+ and K^+. In Na^+-containing buffer the complex is stable with ATPγS, AMP-PNP and partially with ADP. In K^+-containing buffer the complex dissociates with Mg^{2+} alone and neither ATPγS, AMP-PNP nor ADP stabilize the complex.

Dissociation of the mt-Hsp70–Tim44 complex depends on Mge1p

The following protocols were used to remove endogenous nucleotides. First, mitochondria were lysed in K^+-containing buffer and the detergent extracts were immediately desalted by gel filtration in the presence or absence of Mg^{2+} and the indicated nucleotides (Figure 2A). In a second protocol, the mt-Hsp70–Tim44 complex was affinity-purified by immunoadsorption with anti-Tim44 IgG, and Mg^{2+} and nucleotides were subsequently added (Figure 2A). Under both conditions the mt-Hsp70–Tim44 complex was stable in the presence of Mg^{2+}. This suggests that the Mg^{2+}-dependent dissociation of the mt-Hsp70–Tim44 complex in crude mitochondrial lysates was due to endogenous nucleotides which were removed by gel

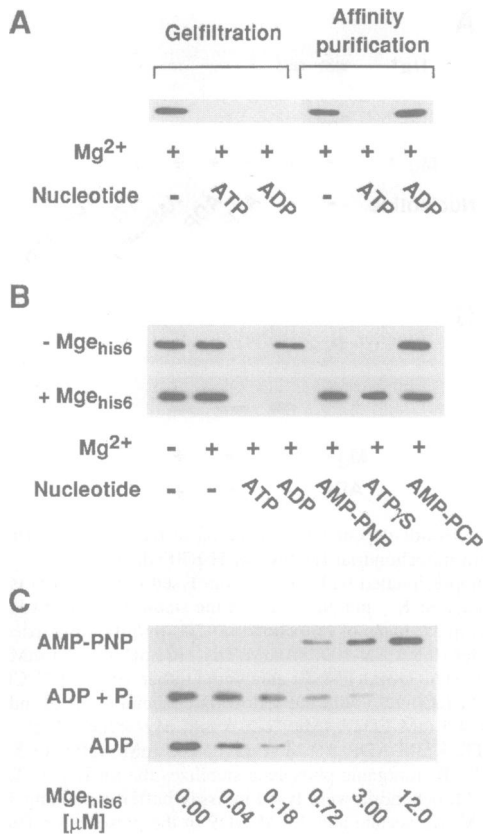

Fig. 2. Mge1p affects the stability of the mt-Hsp70–Tim44 complex in response to nucleotides. Mt-Hsp70 in complex with Tim44 is shown. (**A**) Nucleotide dependence of mt-Hsp70–Tim44 complex dissociation in purified systems. Gel filtration: mitochondria were lysed in assay buffer containing 100 mM KCl in the presence of 3.5 mM Mg-acetate, and ATP (1 mM) or ADP (2.5 mM) when indicated. The mitochondrial extracts were immediately loaded on a Sephadex G-25 M column (1 ml bead volume) and the excluded fractions were subjected to immunoprecipitation with anti-Tim44-antibodies. Affinity-purification: the mt-Hsp70–Tim44 complex was affinity-purified (Materials and methods) and subsequently incubated for 30 min in buffer containing the indicated additions. Mt-Hsp70 in complex with Tim44 was analysed by SDS–PAGE and Western blotting. (**B**) Modulation of the nucleotide-dependent stability of the mt-Hsp70–Tim44 complex by Mge_{his6}. Affinity-purified mt-Hsp70–Tim44 complex was incubated in lysis buffer in the presence or absence of 12 mM Mge_{his6}. Then 5 mM $MgCl_2$ (Mg^{2+}) and the indicated nucleotides were added (100 μM ATP, ADP, FPLC purified AMP-PNP, FPLC purified ATPγS or AMP-PCP). After an incubation at 4°C for 30 min the beads were washed twice with lysis buffer and mt-Hsp70 bound to Tim44 was monitored. (**C**) Dependence of the stability of the mt-Hsp70–Tim44 complex on the concentration of Mge_{his6}. Affinity-purified mt-Hsp70–Tim44 complex was incubated with the indicated concentrations of Mge_{his6} in lysis buffer containing 5 mM $MgCl_2$ and 100 mM KCl in the presence of 250 μM ADP + 4 mM NaCl (ADP), 250 μM ADP + 4 mM NaH_2PO_4 pH 7.4 (ADP+P_i) or 250 μM FPLC purified AMP-PNP (AMP-PNP).

filtration or by affinity-purification of the complex. ATP caused complex dissociation in the gel-filtered extract and after affinity-purification. Interestingly, in the presence of ADP the mt-Hsp70–Tim44 complex dissociated in the gel-filtered extract but it was stable after affinity-purification. Obviously, a high molecular weight factor modulated the response of the mt-Hsp70–Tim44 complex to ADP. This factor was present in the gel-filtered mitochondrial lysate but was lost by affinity-purification of the complex.

We asked whether this factor could be Mge1p. When the mt-Hsp70–Tim44 complex is immunoprecipitated the majority of Mge1p remains in the supernatant and only trace amounts of Mge1p co-purify with the complex (Schneider *et al.*, 1994). To investigate the influence of Mge1p on the stability of affinity-purified mt-Hsp70–Tim44 complex we prepared and used Mge_{his6}, recombinant Mge1p with a hexa-histidine tag. Mge_{his6} had a pronounced effect on the stability of the purified mt-Hsp70–Tim44 complex (Figure 2B). Without added Mge_{his6} the purified complex dissociated in the presence of AMP-PNP and ATPγS but was stable with ADP. In contrast, when Mge_{his6} was added, the complex was stable with AMP-PNP and ATPγS but dissociated in the presence of ADP. The complex was stable with Mg^{2+} alone and with AMP-PCP and it dissociated with ATP, independently of addition of Mge_{his6}. Thus, Mge_{his6} stabilizes the mt-Hsp70–Tim44 complex in the presence of ATP analogues, indicating that it assists in formation of the complex prior to ATP hydrolysis. Mge1p dissociates the complex with ADP, while the stability of the nucleotide-free complex is not affected by Mge_{his6}. This suggests that Mge1p has a function in addition to nucleotide exchange. It directly destabilizes the ADP form of the mt-Hsp70–Tim44 complex which leads to complex dissociation prior to the release of ADP from mt-Hsp70.

We asked what concentrations of Mge1p were necessary to dissociate the mt-Hsp70–Tim44 complex in the presence of ADP or to stabilize the complex in the presence of AMP-PNP. Immunopurified mt-Hsp70–Tim44 complex was incubated with increasing concentrations of Mge_{his6} in the presence of Mg^{2+} and nucleotides. Approximately 0.04–0.18 mM Mge_{his6} were sufficient to significantly destabilize the complex in the presence of ADP and all mt-Hsp70 was released from Tim44 at 0.72 mM Mge_{his6} (Figure 2C). Inorganic phosphate stabilized the Hsp70–Tim44 complex and >3 mM Mge_{his6} was required for complete dissociation in the presence of ADP+P_i. This suggests that Mge1p dissociates the mt-Hsp70–Tim44 complex in the ADP form but not in the ADP+P_i form. At concentrations >0.72 μM, Mge_{his6} stabilized the mt-Hsp70–Tim44 complex in the presence of AMP-PNP. Mge1p comprises ~0.07% of the mitochondrial protein (see below), which corresponds to a concentration in the region of 12 mM; at this concentration the complex was found to dissociate with ADP and to be stable with AMP-PNP. The dissociation of the mt-Hsp70–Tim44 complex in mitochondrial extracts with both ADP and AMP-PNP and its stability with ADP+P_i is presumably due to the lowered concentration of Mge1p. Occupation of the K^+-binding sites in mt-Hsp70 appears to be crucial, as in the presence of Na^+ the mt-Hsp70–Tim44 complex was stable in ADP and AMP-PNP and no modulation of complex stability by Mge_{his6} was observed (not shown). Conditions which preserve or dissociate purified mt-Hsp70–Tim44 complex are summarized in Table I.

In conclusion, in mitochondria the mt-Hsp70–Tim44 complex will form with ATP and dissociate after ATP hydrolysis and P_i release.

The ssc1-3 mutation reduces the affinity of mt-Hsp70 for Mge1p

Mutant forms of mt-Hsp70 have been used to analyse its the role in protein import (Gambill *et al.*, 1993) and to

Table I. Nucleotide-dependent stability of the mt-Hsp70–Tim44 complex

Nucleotide	Mg^{2+}	–Mge		+Mge	
		Na^+	K^+	Na^+	K^+
–	–	+	+	+	+
–	+	+	+	+	+
ATP	+	–	–	–	–
ADP	+	+	+	+	–
ADP+P_i	+	+	+	+	±
AMP-PNP	+	+	–	+	+
ATPγS	+	+	–	n.d.	+
AMP-PCP	+	+	+	n.d.	+

Stability (+) or dissociation (–) of affinity-purified mt-Hsp70–Tim44 complex in response to the indicated nucleotides and nucleotide analogues is summarized. Concentrations used: Mg^{2+}, 5 mM; Na^+, 100 mM; K^+, 100 mM; Mge_{his6}, 12 mM; nucleotides and nucleotide analogues, 100 μM. n.d., not determined.

investigate its interaction with Tim44 (Schneider *et al.*, 1994). In view of the pronounced effect of Mge1p on the mt-Hsp70–Tim44 complex we studied the interaction of Mge1p with mt-Hsp70 in the *ssc1-3* mutant. In this mutant mt-Hsp70 harbours a point mutation in its ATPase domain that leads to a temperature-sensitive growth defect (Gambill *et al.*, 1993).

The mt-Hsp70–Tim44 complex was affinity purified from *ssc1-3* cells in the presence of K^+ and the influence of nucleotides and Mge_{his6} on complex stability was investigated (Figure 3A). In the absence and in the presence of Mge_{his6} the complex between Ssc1-3p and Tim44 was stable with ATP while the mt-Hsp70–Tim44 complex from wild-type yeast dissociated. In the presence of ADP the Ssc1-3p–Tim44 complex was not dissociated by Mge_{his6}. Thus, the *ssc1-3* mutation affects the interaction of mt-Hsp70 with Tim44 without shift to non-permissive temperature.

We asked whether this defect was due to an altered interaction of Ssc1-3p with Mge1p. Mge_{his6} was covalently coupled to BrCN-activated Sepharose and mitochondrial lysates were passed over the affinity resin. In the presence of EDTA, mt-Hsp70 from wild type bound efficiently to the Mge–Sepharose and was subsequently eluted with Mg^{2+} and ATP (Figure 3B). When lysed mitochondria harbouring Ssc1-3p were applied to the Mge–Sepharose the mutant mt-Hsp70 was almost completely recovered in the flow-through fraction, indicating that Ssc1-3p did not bind tightly to Mge_{his6}. Similarly, immunoprecipitation of Ssc1-3p failed to co-precipitate Mge1p from lysed mitochondria (not shown). This demonstrates that the *ssc1-3* mutation reduces the affinity of mt-Hsp70 for Mge1p.

To investigate whether the *ssc1-3* mutation affects the interaction of mt-Hsp70 with nucleotides, mitochondria from mutant and wild-type strains were either pretreated at non-permissive temperature or kept on ice. The mitochondria were lysed and the soluble proteins were applied to ATP–agarose (Figure 3C). Neither without nor with pretreatment at non-permissive temperature did Ssc1-3p bind to ATP–agarose, indicating that the mutation reduces the affinity of the protein for ATP.

When extracts were prepared after exposing *ssc1-3* mitochondria to non-permissive temperature they contained little mt-Hsp70. Instead, Ssc1-3p pelleted after a clarifying spin, indicating that incubation at non-permissive temperature induced aggregation of mutant mt-Hsp70 (Figure 3D).

In conclusion, the *ssc1-3* mutation reduces the affinity of mt-Hsp70 for both nucleotides and Mge1p. As a consequence, the complex between Ssc1-3p and Tim44 exhibits an increased stability with ATP and also with ADP in the presence of Mge1p. Defects in protein import into *ssc1-3* mitochondria after pretreatment at non-permissive temperature (Gambill *et al.*, 1993) might be due to aggregation of mt-Hsp70.

Mge1p levels affect import of folded preprotein

We constructed a yeast strain which overexpressed Mge1p from a multicopy plasmid. The yeast cells were viable and did not exhibit a detectable growth defect on fermentable or non-fermentable carbon sources (not shown). Mitochondria isolated from this strain contained ~30-fold increased levels of Mge1p (Figure 4A). Using purified Mge1p as a reference in Western blotting, we estimated Mge1p to comprise ~0.07% of total mitochondrial protein in wild-type and 2.0% in Mge1p-overexpressing cells. How does Mge1p affect unfolding and import of preproteins into mitochondria? We measured import of the chimeric preproteins pSu9(1–69)-DHFR and pSu9(1–69)-DHFRmut, a variant of pSu9(1–69)-DHFR that carries mutations that destabilize the DHFR domain (Teichmann *et al.*, 1996). The kinetics of import of pSu9(1–69)-DHFR into mitochondria containing increased levels of Mge1p were significantly reduced while those of pSu9(1–69)-DHFRmut were not affected (Figure 4B). Thus, unfolding of folded preproteins on the mitochondrial surface is impeded when high concentrations of Mge1p are present in the matrix.

We analysed whether increased levels of Mge1p affect the interaction of mt-Hsp70 with Tim44 or with the preprotein in transit. Mitochondria harbouring overexpressed levels of Mge1p and control mitochondria were lysed in the presence of EDTA and mt-Hsp70 in complex with Tim44 was determined by co-precipitation with antibodies against Tim44 (Figure 4C). Overexpression of Mge1p did not affect the yield of mt-Hsp70–Tim44 complex. The chimeric preprotein pSu9(1–86)+66-DHFR (Ungermann *et al.*, 1996) was incubated in the presence of methotrexate (MTX) with energized mitochondria from Mge1p-overexpressing and wild-type yeast. The protein accumulated in a membrane-spanning fashion and the processed translocation intermediate exposed a segment of ~36 amino acid residues into the matrix (Ungermann *et al.*, 1996). To determine mt-Hsp70 bound to this segment the mitochondria were lysed and immunoprecipitations with antibodies against Hsp70 were performed (Figure 4D). With mitochondria from the Mge1p-overexpressing strain the amount of mSu9(1–86)+66-DHFR that co-precipitated with mt-Hsp70 was reduced 3-fold. As the formation of the mt-Hsp70–Tim44 complex was not reduced in these mitochondria it seems likely that high levels of Mge1p reduce binding of mt-Hsp70 to the incoming preprotein and/or accelerate the release of bound mt-Hsp70 from the preprotein. This allows retrograde movement of the preprotein chain in the import channel (Schneider *et al.*, 1994; Ungermann *et al.*, 1994).

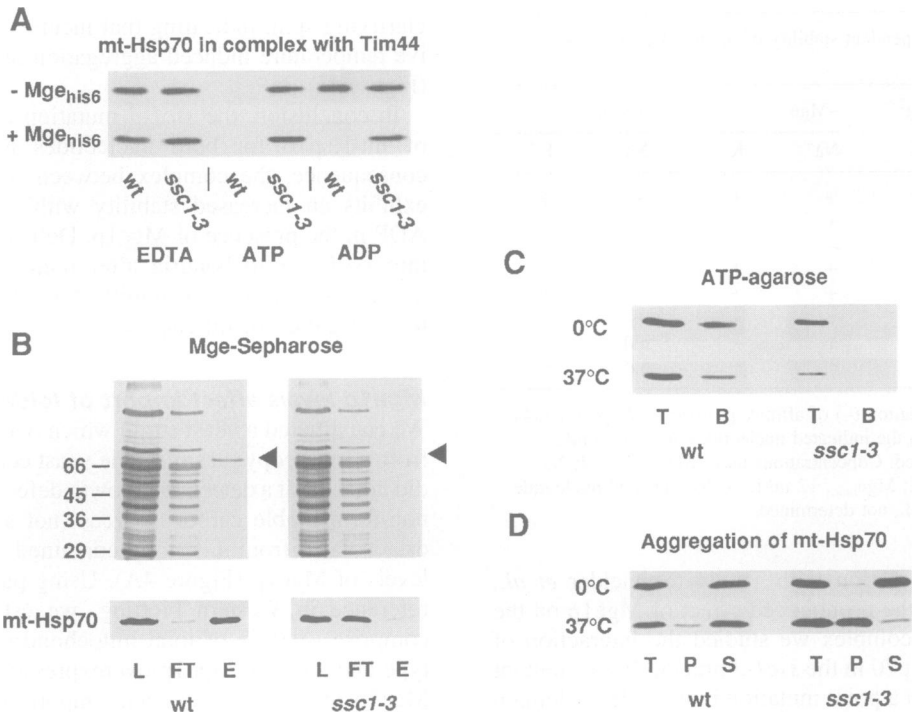

Fig. 3. The *ssc1-3* mutation affects the ATP- and Mge1p-dependent cycle of mt-Hsp70–Tim44 interaction at permissive temperature. (**A**) Ssc1-3p is defective in the nucleotide-dependent dissociation from Tim44. The mt-Hsp70–Tim44 complex was affinity-purified from *ssc1-3* or wild-type (wt) yeast in lysis buffer containing 5 mM EDTA. Subsequently the stability of the complex was monitored in EDTA (5 mM), ATP (5 mM $MgCl_2$, 2 mM ATP) or ADP (5 mM $MgCl_2$, 2 mM ADP) in the presence or absence of Mge_{his6} (3 mM). (**B**) Mt-Hsp70 with the *ssc1-3* mutation does not bind to Mge–Sepharose. Mitochondria were prepared from *ssc1-3* or from wild-type (wt) yeast cells which were grown at 24°C. Mitochondria (500 μg) were lysed in 500 μl 0.5% Triton X-100, 80 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.1 mg/ml α_2-macroglobulin, 20 mM MOPS–KOH pH 7.2. After a clarifying spin (10 min, 25 000 *g*) the extracts were passed four times over an Mge–Sepharose column (0.2 ml bed volume). The resin was washed with 2 ml of buffer and mt-Hsp70 was eluted with buffer containing 5 mM $MgCl_2$ and 5 mM ATP. Aliquots of the load (L), the flow-through fraction (FT) and the eluate (E) were analysed by SDS–PAGE. A Coomassie blue-stained gel is shown in the upper panel. Mt-Hsp70 is indicated by an arrowhead. Lower panel: Western blot with anti-mt-Hsp70 IgG. (**C**) Ssc1-3p does not bind to ATP–agarose. Mitochondrial lysates from *ssc1-3* and wild-type yeast were prepared as described (von Ahsen *et al.*, 1995). Aliquots (200 ml) were incubated for 60 min at 4°C by end-over-end rotation with ATP–agarose (1 mg dry weight). The ATP–agarose beads were washed twice with lysis buffer containing 5 mM $MgCl_2$. Mt-Hsp70 was monitored by SDS–PAGE and Western blotting. (**D**) Ssc1-3p aggregates at 37°C. Mitochondria from *ssc1-3* or wild-type yeast were incubated for 15 min at 37°C (Gambill *et al.*, 1993). Control samples were kept on ice (0°C). Mitochondria were then lysed with 0.1% TX-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 mM MOPS–KOH pH 7.2 and the solubilized mitochondria (0.5 mg/ml) were subjected to a clarifying spin (1 h, 100 000 *g*). Aliquots of the lysed mitochondria (T), the pellet (P) and supernatant (S) of the clarifying spin were analysed by SDS–PAGE and Western blotting with antibodies against mt-Hsp70.

Discussion

Preprotein translocation across the mitochondrial inner membrane is driven by cycles of interaction of mt-Hsp70 with Tim44 and the preprotein in transit. Here, we have investigated the role of the nucleotide exchange factor Mge1p, a co-chaperone of mt-Hsp70, in the import reaction cycle. We show that Mge1p exerts a decisive function at multiple steps of the cycle. Furthermore, the interaction of mt-Hsp70 with Tim44 is influenced by inorganic phosphate. On the basis of our results we propose a reaction cycle which incorporates the roles of both Mge1p and inorganic phosphate (Figure 5): in a productive reaction cycle a complex forms between Tim44 and mt-Hsp70 in the presence of ATP. Formation of this complex is promoted by Mge1p. ATP is hydrolysed by mt-Hsp70 in complex with Tim44 and the inorganic phosphate produced becomes released. Mge1p then facilitates dissociation of the mt-Hsp70–Tim44 complex. Subsequently, Mge1p promotes ADP release from and ATP uptake by mt-Hsp70. The mt-Hsp70 in the ATP form can then engage in a new cycle. This reaction cycle is notably similar to the reaction cycle of bacterial DnaK with DnaJ and GrpE (Szabo *et al.*, 1994; McCarty *et al.*, 1995).

The role of ATP binding and ATP hydrolysis in formation and dissociation of the mt-Hsp70–Tim44 complex has been controversial (Schneider *et al.*, 1994; Glick, 1995; von Ahsen *et al.*, 1995; Horst *et al.*, 1996). It was proposed that ATP is hydrolysed by free mt-Hsp70 rather than in complex with Tim44 (von Ahsen *et al.*, 1995) and that the complex with Tim44 is formed when mt-Hsp70 is in the ADP form (Glick, 1995; von Ahsen *et al.*, 1995). It was further suggested that Mge1p catalyses the release of ADP from the mt-Hsp70–Tim44 complex resulting in a stable, nucleotide-free form of the complex which is then dissociated by binding of ATP to mt-Hsp70 (von Ahsen *et al.*, 1995; Horst *et al.*, 1996). Accordingly, mt-Hsp70 in the ATP form would dissociate from Tim44.

Mge1p is an essential protein which is required for import and folding of proteins in mitochondria (Bolliger *et al.*, 1994; Laloraya *et al.*, 1994; Westermann *et al.*, 1995). It acts as a nucleotide exchange factor for mt-Hsp70 in the mitochondrial matrix. As demonstrated here, Mge1p has an additional function, in that it directly affects the interaction of mt-Hsp70 with Tim44. In addition, it facilitates dissociation of the mt-Hsp70–Tim44 complex in the presence of ADP and promotes complex formation in the presence of ATP.

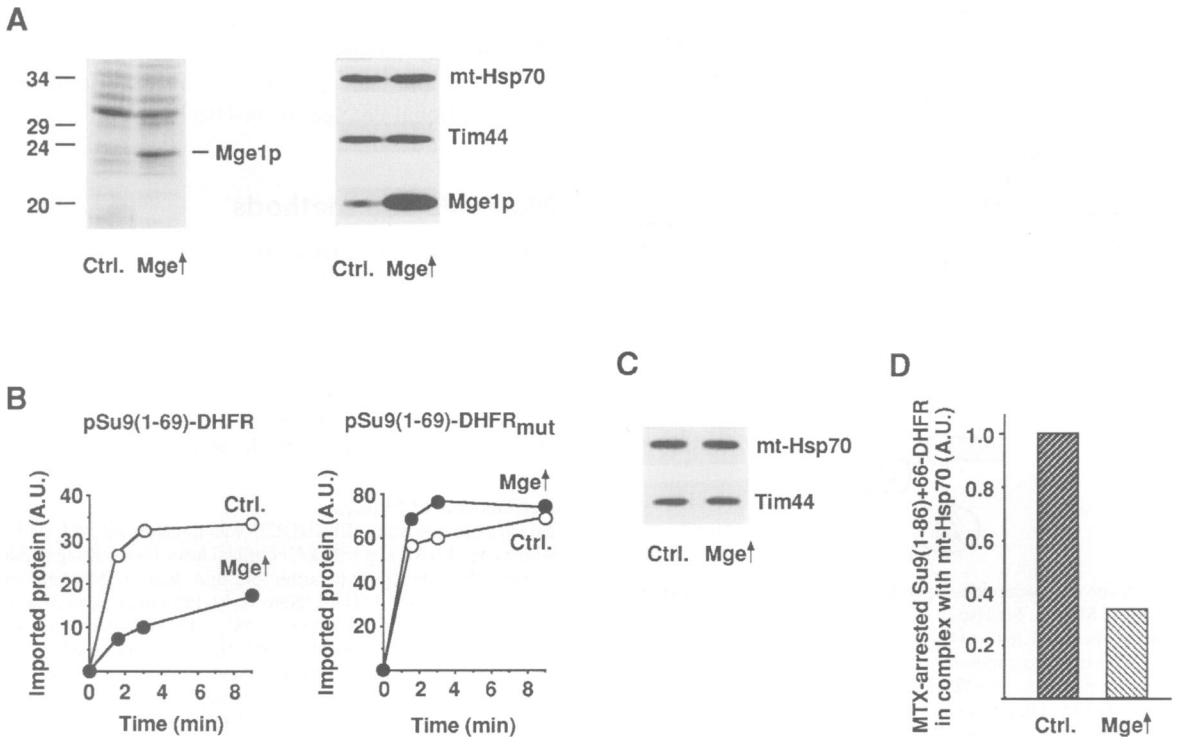

Fig. 4. Overexpression of Mge1p affects protein import. (**A**) Mge1p levels in the matrix. Mitochondria from Mge1p-overexpressing (Mge↑) and control mitochondria from wild-type yeast (Ctrl.) were analysed by SDS–PAGE. Left panel: Coomassie blue stain. Mge1p is indicated by an arrowhead. Right panel: Western blot with indicated antibodies. (**B**) Import defect of mitochondria from Mge1p-overexpressing yeast. Radiolabelled pSu9(1–69)-DHFR or pSu9(1–69)-DHFRmut was incubated at 25°C with energized mitochondria from Mge1p-overexpressing (Mge↑) and control yeast (Ctrl.) in import buffer with an ATP regenerating system. After the times indicated samples were treated with 50 μg/ml proteinase K and analysed by SDS–PAGE and phosphorimaging. The amount of PK-resistant mature protein was plotted versus the time of import at 25°C (A.U., arbitrary units). (**C**) Overexpression of Mge1p does not affect formation of the mt-Hsp70–Tim44 complex. Mitochondria (Mge↑ and Ctrl.) were lysed in assay buffer and the mt-Hsp70–Tim44 complex was precipitated with anti-Tim44 IgGs. The immunoprecipitates were analysed by SDS–PAGE and Western blotting with antibodies against mt-Hsp70 and Tim44. (**D**) Elevated levels of Mge1p reduce mt-Hsp70 bound to preprotein at the import site. The radiolabelled chimeric precursor pSu9(1–86)+66-DHFR was incubated in the presence of MTX with mitochondria from Mge1p-overexpressing (Mge↑) and control yeast (Ctrl.). The mitochondria were then re-isolated and lysed in assay buffer. The supernatant of a clarifying spin was subjected to immunoprecipitation with antibodies against mt-Hsp70. Immunoprecipitates were analysed by SDS–PAGE and the amount of co-precipitated mSu9(1–86)+66-DHFR was determined with a phosphorimager system. The amount of co-precipitated Su9(1–86)+66-DHFR was in the region of 4% of total imported material in the case of control mitochondria.

What is the sequence of events that leads to complex dissociation? We have shown that Mge1p binds to the nucleotide-free form of the mt-Hsp70–Tim44 complex and does not dissociate the complex in this state. Therefore, in the presence of ADP, dissociation of the mt-Hsp70–Tim44 complex must precede ADP release from mt-Hsp70, since otherwise a stable, nucleotide-free complex would be generated. Thus, if in the mitochondria the ADP form of the mt-Hsp70–Tim44 complex is generated in the reaction cycle, this complex will be dissociated by Mge1p prior to the release of ADP from mt-Hsp70. This rules out the possibility that in a productive reaction cycle the mt-Hsp70–Tim44 complex is dissociated by ATP uptake.

How would Mge1p promote formation of the mt-Hsp70–Tim44 complex in the presence of ATP? As ATP is hydrolysed by mt-Hsp70, non-hydrolysable ATP analogues were used to investigate the interaction between Tim44 and mt-Hsp70 in the ATP form. AMP-PCP, an ATP analogue not used in previous studies, stabilizes the complex, suggesting that the complex forms with ATP. The observed dissociation of the nucleotide-depleted mt-Hsp70–Tim44 complex by added ATPγS and AMP-PNP in the absence of effective concentrations of Mge1p most likely reflects the initial binding equilibrium of the complex in the ATP form rather than a forward step in the reaction cycle. The half-lives for dissociation of highly diluted purified mt-Hsp70–Tim44 complex with ATPγS and AMP-PNP are in the range 5–10 min (Horst *et al.* 1996). Therefore this complex is likely to form with these ATP analogues when mt-Hsp70 is present at physiological concentrations (~5000-fold higher) even in the absence of Mge1p. In the presence of Mge1p the complex is stable with all three ATP analogues. Therefore, we conclude that Mge1p promotes formation of the mt-Hsp70–Tim44 complex in the presence of ATP and, after ATP hydrolysis, facilitates dissociation of mt-Hsp70–ADP from Tim44. This is supported by studies on the mutant mt-Hsp70 in the *ssc1-3* strain. The *ssc1-3* mutation reduces the affinity of mt-Hsp70 for ATP and for Mge1p. As a consequence the complex between Ssc1-3p and Tim44 exhibits an increased stability with ATP and is not dissociated by Mge1p in the presence of ADP.

It will be interesting to determine how Mge1p modulates the interaction of mt-Hsp70 with Tim44. It seems conceivable that binding of Mge1p to mt-Hsp70 causes a conformational change in mt-Hsp70 which not only weakens its affinity for nucleotides but also affects the binding site for Tim44.

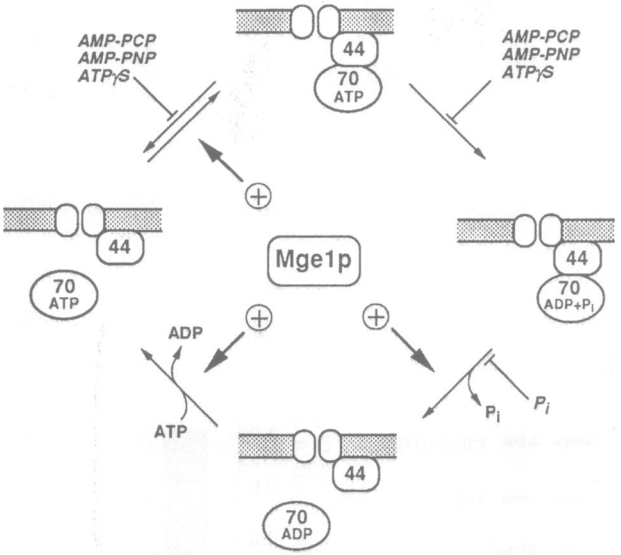

Fig. 5. Model for the nucleotide-dependent reaction cycle of mt-Hsp70 with Tim44 and Mge1p. Mt-Hsp70 (70) in the ATP form associates with membrane-bound Tim44 (44). The complex in the ATP form equilibrates with the free components. Mge1p promotes complex formation in the presence of ATP. ATP is hydrolysed by mt-Hsp70 in complex with Tim44. Then inorganic phosphate is released and the dissociation of mt-Hsp70 in the ADP form from Tim44 is facilitated by Mge1p. Mge1p promotes the release of ADP from free mt-Hsp70 and mt-Hsp70 is recycled by uptake of ATP. The sites of action of ATP analogues and inorganic phosphate (P_i) are indicated.

Mge1p also plays a role in the interaction of mt-Hsp70 with preprotein (Westermann *et al.*, 1995) and is therefore crucial for the mode of protein translocation. It is Hsp70 in the ADP form that binds tightly to substrate proteins (Palleros *et al.*, 1993; Szabo *et al.*, 1994). Thus, in the presence of a preprotein in the import channel mt-Hsp70–ADP will remain associated with the incoming polypeptide after dissociation of the mt-Hsp70–Tim44 complex. Mge1p will sample the various forms of mt-Hsp70 throughout the import reaction cycle and equilibrate nucleotides, which in energized mitochondria means formation of the ATP form of mt-Hsp70. The concentration of Mge1p then determines how long mt-Hsp70 remains bound to a protein substrate, since ATP uptake releases substrate from Hsp70s (Palleros *et al.*, 1993; Szabo *et al.*, 1994). Thus, mt-Hsp70–substrate complexes are stabilized in mitochondria containing reduced levels of functional Mge1p (Laloraya *et al.*, 1995). As shown here, overexpression of Mge1p reduces the rate of import of a preprotein. Presumably, an accelerated release of mt-Hsp70 from the polypeptide mediated by Mge1p promotes backsliding of the chain in the import channel (Ungermann *et al.*, 1994). Consequently, unfolding of folded protein domains such as DHFR on the outside of the mitochondria will be a less-favoured event.

The data presented here are in accordance with the molecular ratchet model for protein translocation (Schneider *et al.*, 1994). mt-Hsp70, Tim44 and Mge1p are indispensable parts of this ratchet with distinct functions. The essential role of Tim44 is to recruit mt-Hsp70 to the import site and keep it there until it has associated tightly with the incoming preprotein. When this has occurred, the key role of Mge1p is to release the mt-Hsp70–preprotein complex from Tim44, which then allows inward movement of the translocating polypeptide chain. The forward movement of the preprotein chain could be driven by thermal motion or directly facilitated by a conformational change in mt-Hsp70.

Materials and methods

Purification of ATP analogues

AMPβ–PNP and ATPγS (Boehringer Mannheim) were purified by anion exchange chromatography on a Mono Q column (1 ml, Pharmacia). The ATP analogues were loaded onto the column in 50 mM MES–KOH pH 6.0 and were eluted with a gradient from 0 to 250 mM NaCl with a flow rate of 0.5 ml/min. Fractions containing AMP-PNP or ATPγS, respectively, were collected and the concentrations of the analogues were determined by their absorbance at 260 nm. AMP-PCP (Boehringer Mannheim) was chromatographically pure.

Purification of Mge_{his6}

Escherichia coli strain BL21(DE3) was transformed with pT7 MGE1B which encodes Mge1p with a C-terminal hexa-histidine tag (Stüber *et al.*, 1990). The Mge1p expression plasmid was a generous gift from N.Heyrovska and F.-U.Hartl (New York). Protein expression was induced with 2 mM IPTG for 4 h at 37°C. The cells were harvested and resuspended in buffer A (300 mM NaCl, 10 mM imidazole, 50 mM Tris–HCl pH 8.0). After treatment with lysozyme (30 min at 4°C and 5 min at 37°C with 1 mg/ml lysozyme, 1 mM PMSF) the cells were sonicated to break the chromosomal DNA (5× 10 s, Branson Sonifier 250) and centrifuged for 30 min at 20 000 r.p.m. in a Beckman JA20-rotor. The supernatant was loaded on a Ni–NTA agarose column (4 ml bed volume). The column was washed with 10 ml buffer A, 25 ml buffer A + 2 mM Mg-acetate, 1 mM ATP and 25 ml of buffer B (300 mM NaCl, 10% glycerol, 50 mM HEPES–KOH pH 7.0). Mge_{his6} was eluted with a linear gradient from 0 to 300 mM imidazole in buffer B. Fractions containing Mge_{his6} were pooled and dialysed against 75 mM NaCl, 10% glycerol, 25 mM HEPES–KOH pH 7.4.

Preparation of Mge–Sepharose

$Mge1_{his6}$ (2.5 mg) was incubated in 100 mM $NaHCO_3$ pH 8.3, with 1 ml CNBr-activated Sepharose 4B (Pharmacia) for 4 h at 4°C. The beads were blocked with 100 mM Tris–HCl pH 8.0, washed with 200 mM NaCl, 30 mM Tris–HCl pH 7.4 and stored in 0.02% NaN_3, 154 mM NaCl, 10 mM Tris–HCl pH 7.4 at 4°C.

Affinity-purification of the mt-Hsp70–Tim44 complex

Mitochondria were incubated for 10 min at 0°C in lysis buffer (0.1% Triton X-100, 250 mM sucrose, 80 mM KCl, 1 mM PMSF, 20 mM MOPS–KOH pH 7.2) containing 5 mM EDTA. The protein concentration was adjusted to 1 mg/ml. After a clarifying spin (10 min, 25 000 *g*) the supernatant was added to anti-Tim44 antibodies prebound to protein A–Sepharose. The samples were incubated at 4°C by end-over-end rotation for 1 h and washed three times in lysis buffer with 0.5 mM EDTA. The complex dissociation was studied in a second incubation for 30 min in lysis buffer with additions as indicated.

Miscellaneous

Saccharomyces cerevisiae strains used were D273-10B (wild-type), PK83 (*ssc1-3*) (Gambill *et al.*, 1993) and YBW36 containing a disruption of the genomic *MGE1* and the multicopy plasmid pBWM23 carrying the *MGE1* gene (Westermann *et al.*, 1995). Affinity-purified antibodies were prepared according to Schneider *et al.* (1994). Import of preproteins into mitochondria, MTX arrest of DHFR fusion proteins and co-immunoprecipitation of translocation intermediates with mt-Hsp70 were performed as described (Ungermann *et al.*, 1994, 1996).

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