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The role of the GrpE homologue, Mge1p, in mediating protein import and protein folding in mitochondria

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Mge1p, a mitochondrial GrpE homologue, has recently been identified in the yeast *Saccharomyces cerevisiae* and a role for this protein in precursor import has been reported. To dissect the molecular mechanism of Mge1p function, conditional *mge1* mutants were constructed. Cells harbouring mutant *mge1* accumulated precursor proteins at restrictive temperature. Both kinetics and efficiency of import were reduced in mitochondria isolated from strains possessing mutant *mge1*. Binding of mitochondrial-Hsp70 (mt-Hsp70) to incoming precursor proteins was abolished at restrictive temperature. Nucleotide-dependent dissociation of mt-Hsp70 from the import component MIM44 was reduced in mitochondria from mutant *mge1* strains. Furthermore, at restrictive temperature an increase of incompletely folded, newly imported protein and enhanced protein aggregation was observed in mitochondria isolated from the mutant strains. We conclude that Mge1p exerts an essential function in import and folding of proteins by controlling the nucleotide-dependent binding of mt-Hsp70 to substrate proteins and the association of mt-Hsp70 with MIM44.

Key words: eukaryotic GrpE homologue/mitochondrial Hsp70/mitochondrial protein import/protein folding

Introduction

Heat shock proteins of the Hsp70 family are essential components in a number of diverse biological processes, such as folding and assembly of proteins, protection against heat damage, protein degradation and translocation of proteins across intracellular membranes (for review see Ellis, 1993; Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Craig *et al.*, 1994; Wickner, 1994). Mitochondrial-Hsp70 (mt-Hsp70), encoded by the *SSC1* gene in *Saccharomyces cerevisiae*, plays an essential role in protein import into the mitochondrial matrix (for review see Stuart *et al.*, 1994a). Temperature-sensitive mutants of mt-Hsp70 accumulate incompletely translocated precursor proteins across the two mitochondrial membranes (Kang *et al.*, 1990; Gambill *et al.*, 1993; Voos *et al.*, 1993). Depletion of ATP in the mitochondrial matrix has a similar effect on protein import. In this case, matrix-destined precursor proteins are unable to undergo complete translocation across the inner membrane, resulting in membrane spanning translocation intermediates which partially accumulate in the intermembrane space (Hwang *et al.*, 1991; Rassow and Pfanner, 1991; Jascur *et al.*, 1992). ATP-dependence of protein import has been demonstrated to be linked to mt-Hsp70 function (Cyr *et al.*, 1993; Stuart *et al.*, 1994b), thus, the ATPase activity of mt-Hsp70 plays an indispensable role in the transfer process. Unidirectional translocation is achieved by the nucleotide-dependent co-operation of mt-Hsp70 with a component of the inner membrane, MIM44 (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). ATP hydrolysis has been proposed to dissociate the mt-Hsp70–MIM44 complex, and the nucleotide-dependent complex formation and dissociation is supposed to facilitate precursor import by a molecular ratchet-like mechanism (Schneider *et al.*, 1994). In addition, a nucleotide-dependent conformational change of mt-Hsp70 and/or MIM44 could assist unfolding of domains outside the mitochondria by exerting a pulling force on the translocating polypeptide (Schneider *et al.*, 1994; Glick, 1995). In both cases, mt-Hsp70 has to undergo cycles of ATP hydrolysis and exchange of ADP versus ATP.

The molecular mechanism of Hsp70 function has been analysed in some detail with the *Escherichia coli* homologue DnaK. Conformational changes of DnaK dependent on the bound nucleotide allow association with, and release from, substrate proteins. This cycle of Hsp70 interaction with substrate protein is regulated by the cohort chaperones DnaJ and GrpE. DnaJ stimulates the ATPase activity of DnaK (Liberek *et al.*, 1991) and might also have a targeting function for DnaK binding to unfolded substrate protein (Wickner *et al.*, 1991; Langer *et al.*, 1992). GrpE acts as a nucleotide exchange factor for DnaK (Liberek *et al.*, 1991) and stably interacts with a conserved loop in the ATPase domain of DnaK (Buchberger *et al.*, 1994). A model put forward by Szabo *et al.* (1994) suggests that DnaK in the ATP form is capable of binding substrate protein. ATP hydrolysis is then stimulated by substrate binding *per se* and/or by the aid of DnaJ. DnaK in the ADP-bound form has a higher affinity for already bound substrate protein. Substrate release is finally facilitated by GrpE which converts DnaK back to the ATP-bound form which in turn has a lower affinity towards bound substrate protein. On the other hand, Palleros *et al.* (1993) suggested a cycle in which the ADP form of Hsp70 is the species competent for substrate binding. Exchange of bound ADP for ATP, mediated by the GrpE protein, converts DnaK into the low affinity form towards substrate protein resulting in its release. DnaJ-stimulated ATP hydrolysis leads then to DnaK–ADP, the substrate binding-competent form.

Several DnaJ homologues have been identified in eukaryotic cells (for review see Caplan *et al.*, 1993; Silver

and Way, 1993). However, the first eukaryotic homologue of the bacterial GrpE protein has only very recently been discovered in mitochondria from *S.cerevisiae* (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Laloraya *et al.*, 1994). In contrast to the gene encoding the mitochondrial DnaJ homologue, *MDJ1* (Rowley *et al.*, 1994), the gene encoding mitochondrial GrpE, *MGE1* (also termed *YGE1*), is essential. Cells depleted of Mge1p accumulate mitochondrial precursor, pointing to a role for Mge1p in mitochondrial protein import (Laloraya *et al.*, 1994; Nakai *et al.*, 1994). Interaction of Mge1p with translocating polypeptides via mt-Hsp70 has been demonstrated biochemically (Voos *et al.*, 1994).

It is presently not clear at which step Mge1p is required for import of precursor proteins. Since Mge1p is an essential protein, functional analysis by gene disruption is not possible. In order to study the role of the mitochondrial GrpE homologue in precursor import and in other processes involved in mitochondrial biogenesis, conditional mutant alleles of *MGE1* were isolated. Functional analysis of these mutant forms of Mge1p revealed that this protein is required for import by providing mt-Hsp70 in a form which is competent for substrate binding. Mutations in Mge1p not only resulted in a defect in precursor import, but also in deficiency in the folding of newly imported proteins. This points to a general role for Mge1p in modulating mt-Hsp70 function. Our results shed light on both the molecular function of Mge1p and on the mechanism of driving unidirectional transfer of preproteins across mitochondrial membranes.

Results

Isolation of temperature-sensitive mge1 mutants

The *MGE1* gene was obtained by PCR amplification of genomic DNA from *S.cerevisiae*. After cloning, the amplified fragment was subjected to hydroxylamine mutagenesis. The mutagenized DNA was introduced into a *S.cerevisiae* strain with a chromosomal null allele of *MGE1* and a plasmid providing the essential gene in *trans*. Transformants were reselected by plasmid shuffling in order to obtain cells exclusively possessing the plasmid with mutagenized *MGE1* (for details see Materials and methods). Four mutant strains (*mge1-2* to *mge1-5*) were obtained which were temperature-sensitive for growth at 37°C. At 25 and 30°C, the growth of all mutant strains was indistinguishable from that of wild-type on both fermentable and non-fermentable carbon sources. At 37°C, the mutant strain *mge1-2* did not grow at all, however, mutant strains *mge1-3*, *mge1-4* and *mge1-5* showed some residual growth (Figure 1). The protein level of Mge1p present in the mutant strains was tested by immunoblotting. The amount of Mge1p in mitochondria from strain *mge1-2* was reduced to ~20% of wild-type, whereas it was not changed in mitochondria from strains *mge1-3*, *mge1-4* and *mge1-5* (data not shown).

DNA sequence analysis revealed that each of the ts-mutant alleles possessed a single amino acid exchange (see legend to Figure 1). This was in addition to an exchange introduced into the wild-type gene during PCR amplification. Since the PCR-generated allele fully complemented the *MGE1* deletion strain, it can be regarded as functionally equivalent to the *MGE1* wild-type gene, and cells with this allele are referred to as wild-type. Some of the exchanges present in the ts-alleles were close to conserved residues or within conserved domains defined for bacterial GrpE proteins (Wu *et al.*, 1994), however, no mutant allele with an exchange of a highly conserved residue was obtained.

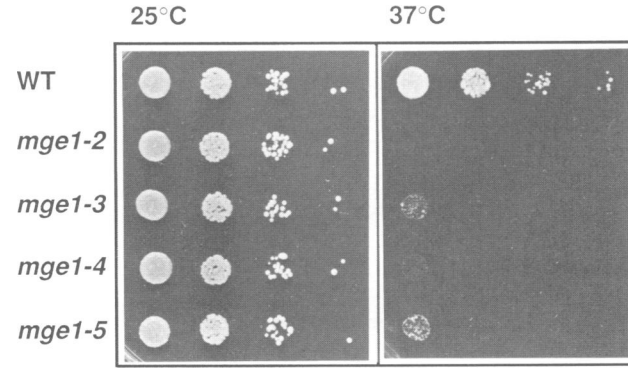

Fig. 1. Growth phenotypes of strains carrying *mge1* ts-alleles in comparison with wild-type cells at restrictive and permissive temperature. *mge1* ts-strains and the isogenic wild-type strain were grown to mid-logarithmic phase at 25°C in liquid YPD medium. Ten-fold serial dilutions of these cultures were spotted onto YPD plates and incubated for 3 days at 25 or 37°C. In addition to a mutation introduced during PCR amplification of the wild-type gene (T_{199} to A), the ts-alleles carry the following amino acid exchanges: *mge1-2*, A_{81} to D; *mge1-3*, A_{134} to V; *mge1-4*, E_{123} to K and *mge1-5*, T_{102} to I. Cells with the PCR-generated *MGE1* allele expressed from the same plasmid as the ts-alleles were used as a wild-type control in all experiments.

Cells with mutant Mge1p accumulate mitochondrial precursor proteins in vivo

The four temperature-sensitive *mge1* mutant strains were analysed for impaired mitochondrial import *in vivo* at permissive and restrictive temperature. The strains were grown at 25°C to mid-logarithmic phase, then half of each culture was maintained at permissive temperature whereas the other half was shifted to 37°C for 30 min. Total cell extract was analysed by SDS–PAGE, Western blotting and immunodecoration with antiserum against Hsp60. When the cells had been maintained at 25°C, all Hsp60 was found as the mature form. In contrast, immunoblots from mutant cell cultures shifted to 37°C showed accumulation of Hsp60 in the precursor form, whereas only a trace of Hsp60 precursor could be detected in extracts from wild-type cells exposed to 37°C (Figure 2). These results indicate that the mutations in the *mge1* alleles cause a defect in mitochondrial protein import.

Protein import into isolated mitochondria from mge1 mutant strains is impaired

To investigate the role of Mge1p in import of precursor proteins in more detail, mitochondria were isolated from the strains harbouring the mutant alleles *mge1-2* to *mge1-5*, and *in vitro* protein import was performed. The import efficiency of two mitochondrial precursor proteins was tested, $pF_1\beta$ and pSu9(1–79)–DHFR, a fusion protein consisting of the N-terminal 79 amino acids of *Neurospora crassa* F_0-ATPase subunit 9 preceding the complete mouse dihydrofolate reductase (C.Ungermann, D.M.Cyr and W.Neupert, unpublished). Mitochondria were pre-incub-

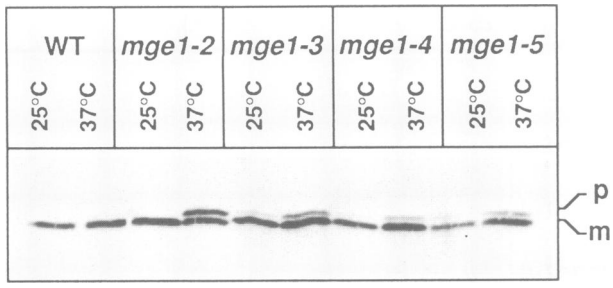

Fig. 2. Strains possessing conditional *mge1* alleles accumulate Hsp60 precursor after shift to non-permissive temperature. Wild-type cells and ts-mutant strains were grown in liquid YPGal medium at 25°C to mid-logarithmic phase. The cultures were then split into two parts of which one was maintained at 25°C, while the other one was shifted to 37°C for 30 min. Cells were then collected and cell extracts were prepared according to Brandt (1991). After SDS–PAGE and transfer to nitrocellulose, cellular protein was reacted with antiserum against mitochondrial Hsp60. p, precursor; m, mature form.

ated for 15 min at either 25 or 37°C. Import was then performed at either permissive temperature (25°C) or restrictive temperature (37°C). At 25°C, import efficiency of both precursor proteins was reduced in mitochondria from the mutant strains in comparison with wild-type. However, in all cases the import defect was stronger at 37 than at 25°C (Figure 3). In general, import of $pF_1\beta$ was more severely reduced than that of pSu9(1–79)–DHFR. These results demonstrate that Mge1p is needed for efficient precursor import *in vitro*, and that the phenotype of the mutants can be induced in isolated organelles. Possible explanations for the more pronounced effect of the mutations at elevated temperature are: (i) the mutant Mge1 proteins are temperature-sensitive gene products undergoing conformational changes at restrictive temperature and (ii) the effect of the mutation becomes manifest at elevated temperature since more functional Mge1p is needed under these conditions. Since import is already reduced at permissive temperature, the latter possibility seems to be more likely. As a result of this analysis the two mutants *mge1-2* and *mge1-3* were chosen for further analysis, the first one displaying the strongest phenotype, the second one possessing wild-type Mge1p level.

The severity of the import deficiency of mitochondria harbouring mutant Mge1p depended on the precursor protein investigated. It is conceivable that a different requirement for mt-Hsp70 exists for pre-proteins depending on the degree of folded structures in the cytosol and/or their different lengths.

We asked whether the import defect was due to a decrease in import efficiency or to slower import kinetics. pSu9(1–79)–DHFR was imported into mitochondria from wild-type and the mutants *mge1-2* and *mge1-3* for different time periods at permissive or restrictive temperature. When import was performed at 37°C and compared with import at 25°C, both import kinetics and efficiency were reduced by a factor of ~3 (Figure 4). Furthermore, in *mge1-2* mutant mitochondria a significant proportion of the mature-sized form remained protease-sensitive after 10 min import at 25°C. This effect was even more pronounced at 37°C, where ~30% of pSu9(1–79)–DHFR matured by the mitochondrial processing peptidase remained accessible to protease. Thus, *mge1-2* mutant mitochondria tend to accumulate translocation intermediates spanning both

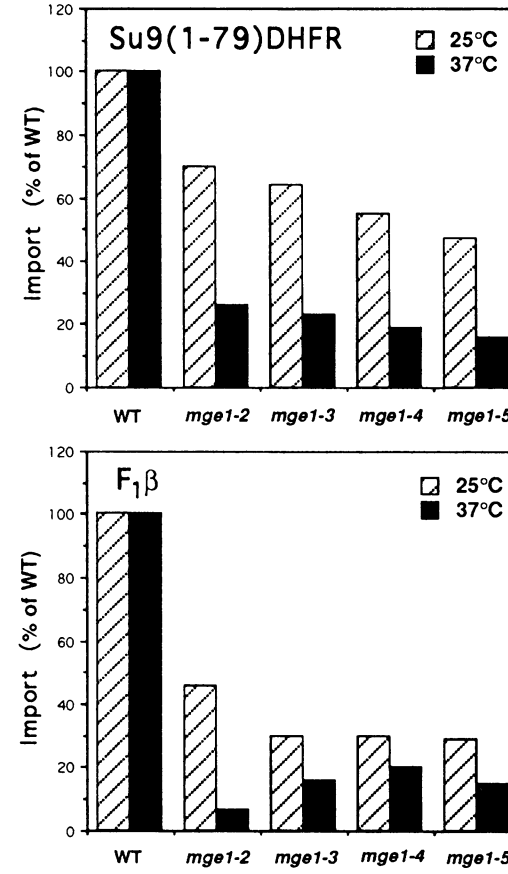

Fig. 3. Import efficiency is reduced in mitochondria from *mge1* mutant strains. Import of radiolabelled pSu9(1–79)–DHFR and $pF_1\beta$ was performed for 10 min at 25 or 37°C as described in Materials and methods. After import, mitochondria were re-isolated by centrifugation, non-imported protein was digested by protease and mitochondrial protein analysed by SDS–PAGE, fluorography and densitometry scanning. The amount of protease-protected mature form is shown. The numbers obtained with wild-type mitochondria were set to 100% for the respective temperature.

membranes. To elucidate the mechanism by which these spanning intermediates arise, further experiments will be required.

Taken together, the results demonstrate that as soon as Mge1p function is compromised, precursor import into isolated mitochondria is impaired, indicating an essential role of Mge1p in the translocation process.

Mge1p is required for the interaction of mt-Hsp70 with incoming precursor proteins

To investigate the molecular basis for the import deficiency displayed by mitochondria with mutant Mge1p, the interaction of mt-Hsp70 with translocating polypeptide chains was analysed. Recently it has been shown that upon depletion of matrix ATP, retrograde movement of arrested translocation intermediates in the translocation channel takes place, resulting in their eventual release into the medium. This has been attributed to the inability of mt-Hsp70 to efficiently bind precursor under these conditions (Ungermann *et al.*, 1994). We tested whether the interaction of mt-Hsp70 with incoming precursor proteins is dependent on the function of Mge1p. During import at permissive or non-permissive temperature, pSu9(1–79)–DHFR was arrested as a membrane spanning translocation

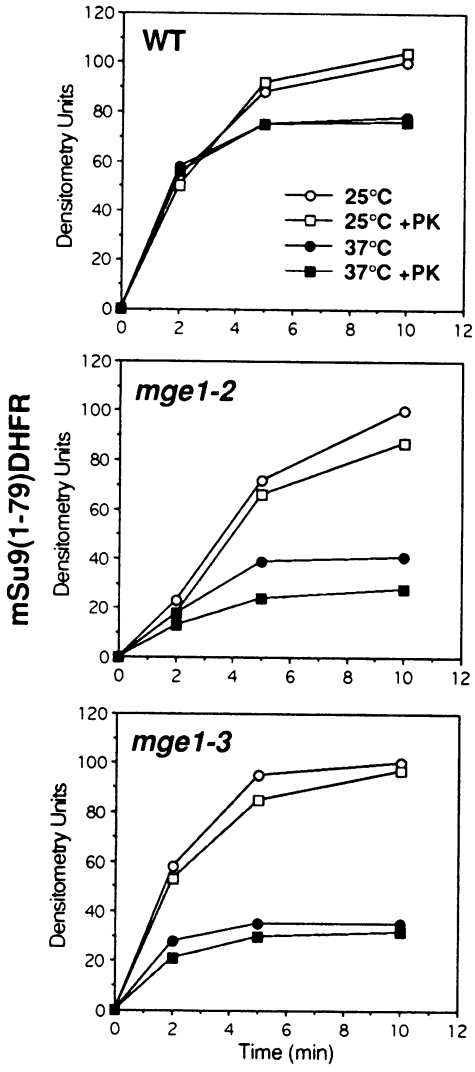

Fig. 4. Mitochondria from *mge1* mutant strains exhibit both reduced import efficiency and reduced import kinetics at restrictive temperature. pSu9(1–79)–DHFR was imported into mitochondria from wild-type and *mge1-2* and *mge1-3* mutant strains at 25 (open symbols) or 37°C (closed symbols). After different times of import samples were removed and import was stopped with valinomycin. Samples were then split into two parts of which one remained untreated (circles) while the other one was subjected to proteinase K treatment (squares). Quantitative analysis was performed after separation of mitochondrial protein by SDS–PAGE, subsequent fluorography and densitometry scanning of the mature form. For each strain the value for 10 min import at 25°C without protease treatment was set to 100.

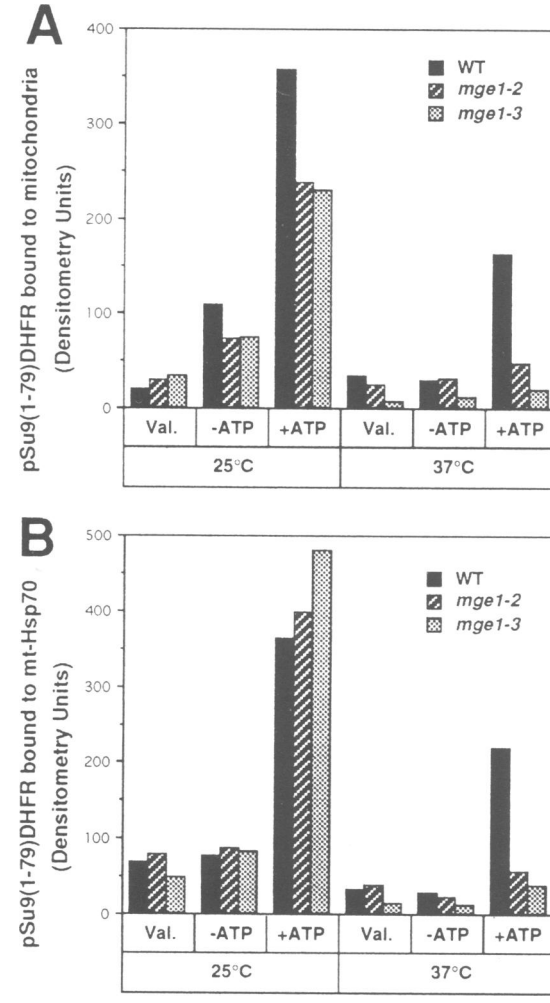

Fig. 5. mt-Hsp70 association of precursor proteins arrested in transit is reduced in mitochondria with impaired Mge1p function. (**A**) MTX-arrested pSu9(1–79)–DHFR was imported into mitochondria at 25 or at 37°C. Before import, mitochondria were either pre-incubated with valinomycin (Val.), with oligomycin and apyrase (−ATP) or in the presence of an ATP-regenerating system (+ATP). The amount of precursor associated with mitochondria was determined after re-isolation of mitochondria, separation of mitochondrial protein by SDS–PAGE, fluorography and laser densitometry. The panel displays absolute densitometry units. (**B**) In parallel, co-immunoprecipitation with antiserum against mt-Hsp70 was carried out. As above, quantitative analysis of mt-Hsp70-associated material was achieved by laser densitometry. The panel displays absolute densitometry units. With pre-immune serum no co-immunoprecipitation was observed (not shown).

intermediate by stabilizing the DHFR domain with methotrexate (MTX), and the amount of precursor protein associated with mitochondria was assessed. As a control for non-specific binding of precursor protein to the surface of mitochondria, one sample was incubated in the presence of valinomycin to destroy the membrane potential. As a control for mt-Hsp70-independent association, a second sample was incubated in the presence of NADH, oligomycin and apyrase in order to maintain the membrane potential, but to deplete the matrix of ATP. A third sample was incubated with NADH, ATP and an ATP-regenerating system, to allow the N-terminal part of the precursor protein to enter the matrix and to be held by mt-Hsp70. Upon import at 25°C, specific interaction of translocation intermediates with mitochondria was comparable in wild-type and mutant mitochondria when the level of matrix ATP was high. Without a membrane potential or at low matrix ATP, only very little interaction was observed. Upon import at 37°C, binding of precursor protein was only found in wild-type mitochondria, indicating that the ATP-dependent interaction of precursors with the import machinery of the inner membrane cannot occur when functional Mge1p is lacking (Figure 5A). To confirm specific interaction of precursors with mt-Hsp70, co-immunoprecipitation using antibodies against mt-Hsp70 was carried out. pSu9(1–79)–DHFR was found to interact with mt-Hsp70 only in the presence of a membrane potential and a high level of matrix ATP. Such an interaction was not observed with mitochondria from the *mge1* mutant strains at non-permissive temperature (Figure 5B).

These results indicate that functional Mge1p is needed to enable mt-Hsp70 to efficiently bind to precursor proteins entering the mitochondrial matrix. Since a low matrix ATP level, or the lack of functional Mge1p, have the same effect on the interaction of mt-Hsp70 with precursor protein, it seems very likely that Mge1p acts during import by modulating the ability of mt-Hsp70 to bind substrate proteins by affecting the equilibrium of the nucleotide bound to mt-Hsp70.

Mge1p modulates the nucleotide-dependent complex formation of mt-Hsp70 and MIM44

First, we asked whether the mutations in the Mge1 proteins of the ts-mutants result in an altered ability of Mge1p to interact with mt-Hsp70. Co-immunoprecipitation of Mge1p with antibodies against mt-Hsp70 was carried out. A reduction of the amount of Mge1p found in complex with mt-Hsp70 was detected with *mge1-3* mitochondria, indicating a decreased affinity of the mutant protein for mt-Hsp70 (Figure 6A). Taking into account the reduced level of Mge1p in *mge1-2* mitochondria, the affinity of the mutant protein for mt-Hsp70 was not altered (Figure 6A), however, the absolute amount of Mge1p complexed with mt-Hsp70 was also reduced in this mutant.

Unidirectional translocation of precursor proteins into the mitochondrial matrix has recently been postulated to be facilitated by cycles of interaction of mt-Hsp70 with the inner membrane protein MIM44 which depend on ATP hydrolysis (Schneider *et al.*, 1994). Therefore, if Mge1p affects the nucleotide loading of mt-Hsp70, the stability of the mt-Hsp70–MIM44 complex might be altered in the *mge1* mutants. Association of mt-Hsp70 with MIM44 in intact mitochondria was investigated by co-immunoprecipitation using antibodies against mt-Hsp70. Prior to lysis, mitochondria were pre-incubated at 25 or 37°C in the presence of ATP. Lysis was then performed either in the presence of apyrase and EDTA or without any additions. Thus, hydrolysis of endogenous ATP is allowed to proceed during lysis under the latter conditions, whereas this process is halted under the former conditions, since apyrase hydrolyses the free ATP and EDTA inhibits the hydrolysis of ATP bound to mt-Hsp70. When ATP hydrolysis was inhibited during lysis, efficient co-immunoprecipitation of MIM44 with mt-Hsp70 was obtained with wild-type and both *mge1* mutant mitochondria (Figure 6B). Under the conditions applied, the amount of co-precipitated material was not affected by the pre-incubation temperature. However, in comparison with wild-type mitochondria, more MIM44 was co-precipitated in the case of *mge1-3* mitochondria. This result indicates that the *mge1-3* mutation leads to an enhanced amount of mt-Hsp70 associated with MIM44. When ATP hydrolysis was not inhibited during lysis, no co-immunoprecipitation of MIM44 with mt-Hsp70 was obtained with mitochondria from wild-type or the *mge1-2* mutant. In contrast, in *mge1-3* mitochondria a considerable amount of MIM44 was found associated with mt-Hsp70. Moreover, pre-incubation at 37°C led to an increase of the co-precipitated material (Figure 6B). This finding implies that the ATP-driven dissociation of the mt-Hsp70–MIM44 complex is impaired in *mge1-3* mitochondria.

Next, a titration of the ATP concentration required for dissociation of the mt-Hsp70–MIM44 complex was performed after lysis of mitochondria. With wild-type mitochondria, a concentration of 30 μM ATP at 0°C or 3 μM ATP at 37°C led to almost complete dissociation of the mt-Hsp70–MIM44 complex. In contrast, higher ATP concentrations were required to lead to complex dissociation with extracts from both mutant mitochondria at 0°C. This is probably due to an increased amount of pre-existing complex in these mitochondria at 0°C. At

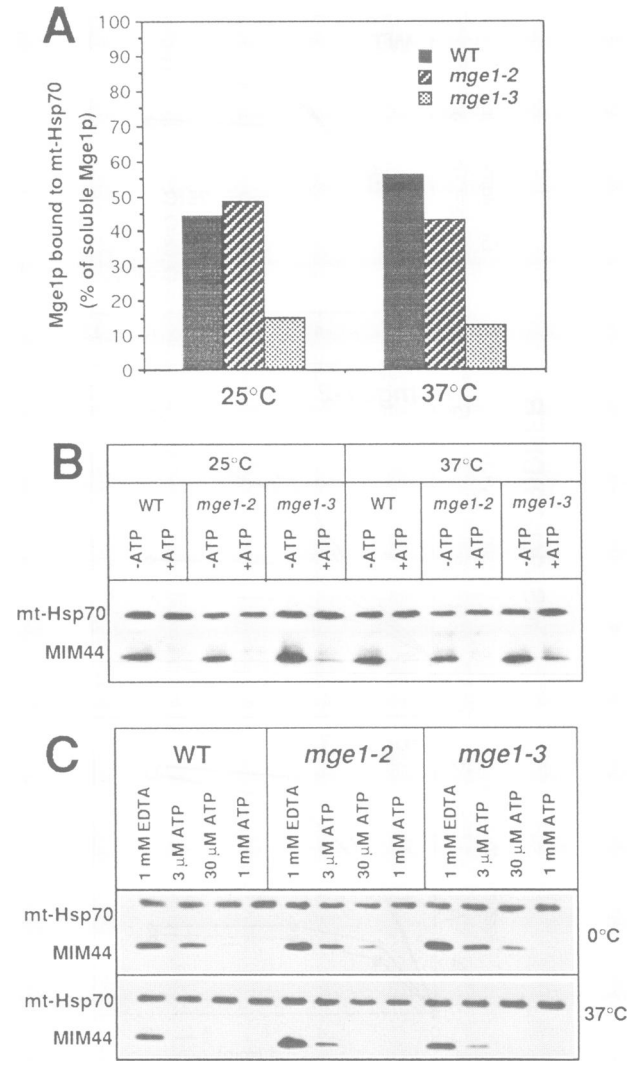

Fig. 6. Affinity of the mutant Mge1 proteins for mt-Hsp70 and nucleotide-dependent mt-Hsp70–MIM44 complex formation in wild-type and *mge1* mutant mitochondria. (**A**) The affinity of Mge1p to mt-Hsp70 was tested by co-immunoprecipitation applying antibodies against mt-Hsp70. The percentage of co-immunoprecipitated material was expressed as the percentage of total Mge1p present in the supernatant before co-immunoprecipitation. (**B**) Pre-existing mt-Hsp70–MIM44 complex was tested by co-immunoprecipitation with antibodies against mt-Hsp70. Mitochondria were incubated in import buffer containing 2.5 mM NADH and 1 mM ATP for 15 min at either 25 or 37°C. Lysis was performed for 15 min on ice either in lysis buffer alone with endogenous ATP (+ATP), or in lysis buffer containing 40 U/ml apyrase to hydrolyse free ATP and 10 mM EDTA to inhibit hydrolysis of ATP bound to mt-Hsp70 during lysis (–ATP). Co-immunoprecipitation was carried out as described in Materials and methods. (**C**) The nucleotide-dependent dissociation of mt-Hsp70 from MIM44 was tested by performing the lysis at 0 or 37°C either in the presence of 1 mM EDTA or increasing concentrations of ATP as indicated. Co-immunoprecipitation was carried out as described in Materials and methods.

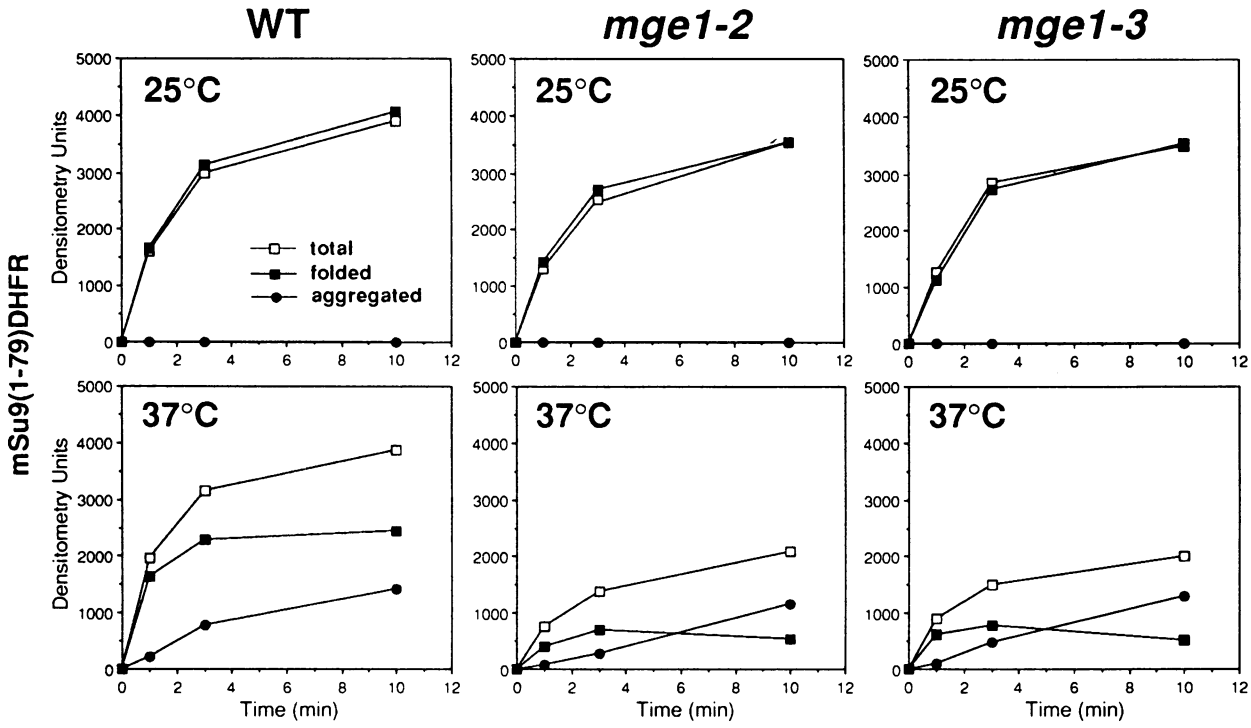

Fig. 7. Folding of newly imported protein is impaired in mitochondria from mutant *mge1* strains at restrictive temperature. pSu9(1–79)–DHFR was imported into mitochondria from wild-type and mutant strains *mge1-2* and *mge1-3* at 25 (upper panels) or 37°C (lower panels). At the time points indicated, samples were removed and non-imported precursor protein was digested with trypsin. After lysis and centrifugation, half of both the pellet and the supernatant was subjected to proteinase K treatment whereas the other half remained untreated (for details see Materials and methods). After SDS–PAGE and fluorography, the amount of mature DHFR in each fraction was quantified by laser densitometry. The panels display absolute densitometry units of mature Su9(1–79)–DHFR.

37°C the amount of pre-existing complex in *mge1-3* mitochondria was comparable with wild-type. Still, in this mutant higher concentrations of ATP were required for complex dissociation (Figure 6C).

In summary, mutations in Mge1p lead to an increase and/or stabilization of the mt-Hsp70–MIM44 complex, probably because mt-Hsp70 cannot efficiently be recycled to the ATP-bound form which after ATP hydrolysis can dissociate from MIM44.

Mge1p is required for the folding of newly imported proteins

A possible function of Mge1p may also exist beyond precursor protein import as it might be involved in folding of proteins after translocation. To investigate this, pSu9(1–79)–DHFR was imported at permissive or restrictive temperature into mitochondria from wild-type and the mutant strains *mge1-2* and *mge1-3*. After various time periods samples were removed and the folding state of DHFR was tested by its solubility and protease resistance. When import was performed at 25°C, the imported material was completely soluble and folded in mitochondria from all strains (Figure 7). When import was performed for 10 min at 37°C, in wild-type mitochondria ~65% of the mature protein was folded and ~35% was found in aggregates. In the mutant mitochondria, the total amount of imported mSu9(1–79)–DHFR was decreased at 37°C because of the import defect. Only ~25% was folded, whereas 55–65% was found in aggregates. Moreover, the amount of soluble mature protein slightly declined over time in the mutant mitochondria (Figure 7). In contrast to wild-type, ~10–20% of mature DHFR was soluble but protease-sensitive in the mutant mitochondria after 10 min at elevated temperature. This species could represent imported material which is still bound to chaperone proteins. It should be noted that all imported protein was present in the matrix space as demonstrated by subfractionation of mitochondria, ruling out the possibility that incomplete translocation might be responsible for the lack of folding (data not shown).

These findings indicate that besides being involved in precursor protein import, Mge1p also plays a role in later steps leading eventually to folded protein. In the absence of functional Mge1p at 37°C, the folding of newly imported DHFR by the mt-Hsp70 machinery might be compromized and/or newly folded protein could be destabilized by elevated temperature and refolding might be impaired. The observation that the amount of folded protein declines with time in mitochondria harbouring mutant Mge1p would favour the latter explanation.

Discussion

The binding of mt-Hsp70 to unfolded pre-proteins entering the matrix appears to be a key reaction conferring unidirectionality on the reversible sliding of the polypeptide chain in the translocation channel (Ungermann *et al.*, 1994). Cycles of binding to, and release from, MIM44 are believed to target mt-Hsp70 to the incoming polypeptide chain and to allow its stepwise progression into the matrix (Schneider *et al.*, 1994). The interactions with both substrate protein and MIM44 are regulated by the

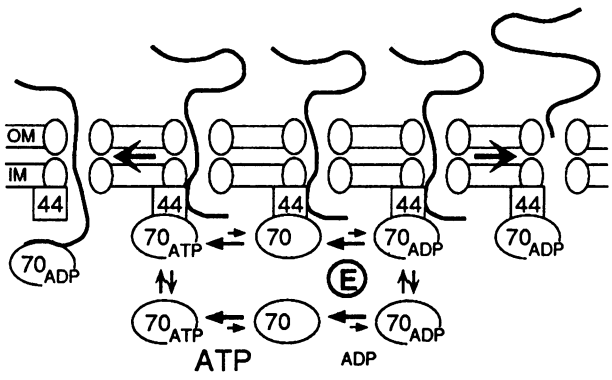

Fig. 8. Model for the action of Mge1p during import. The left side shows the interaction of precursor protein with MIM44 and mt-Hsp70 under conditions where the equilibrium of the nucleotide bound to mt-Hsp70 is shifted to the ATP form, i.e. formation of a productive mt-Hsp70–MIM44 complex; the right side shows the situation with the equilibrium towards the ADP form of mt-Hsp70, i.e. formation of a non-productive mt-Hsp70–MIM44 complex. The Mge1p-mediated nucleotide exchange could occur on both free mt-Hsp70 and mt-Hsp70 in complex with MIM44. See text for further details. OM, mitochondrial outer membrane; IM, mitochondrial inner membrane; 44, MIM44; 70, mt-Hsp70; E, Mge1p.

nucleotide bound to mt-Hsp70. Mge1p, a homologue of bacterial GrpE, which has been described as a nucleotide exchange factor for DnaK (Liberek *et al.*, 1991), can be expected to play an important role in the mt-Hsp70–MIM44 cycle. Here we report on the analysis of conditional mutants of Mge1p and assign an essential role for this component in mitochondrial biogenesis.

At which step of protein import does Mge1p precisely function? According to our previously published working hypothesis (Schneider *et al.*, 1994), mt-Hsp70 in the ATP-bound form is capable of taking over the translocating polypeptide chain from MIM44. Upon ATP hydrolysis, the complex dissociates and mt-Hsp70 in the ADP-bound form is supposed to have a higher affinity towards the pre-protein, thereby preventing backwards movement of the polypeptide chain (Figure 8, left side). After a new cycle of mt-Hsp70 binding to a more C-terminal segment of the pre-protein, a stepwise translocation by a molecular ratchet-like mechanism can be envisioned. This is energetically driven by ATP hydrolysis coupled to the nucleotide cycle-dependent conformational changes of mt-Hsp70.

Also, the ADP-bound form of mt-Hsp70 may have a certain affinity for MIM44 (Kronidou *et al.*, 1994). In this case, translocating precursors may bind to MIM44, however, transfer of the polypeptide chain to mt-Hsp70 in the ADP-bound form would be impaired, and the precursor would diffuse backwards or even out of the translocation channel (Figure 8, right side). This latter situation very likely predominates in mitochondria lacking functional Mge1p. In fact, the mt-Hsp70–MIM44 complex was formed in *mge1* mutant mitochondria, however, higher concentrations of ATP were required to lead to complex dissociation. This finding is in agreement with the interpretation that in mutant mitochondria the equilibrium is shifted to mt-Hsp70 in the ADP-bound form, where dissociation from MIM44 cannot be triggered by hydrolysis of ATP. This complex in turn is non-productive, since the ATP-bound form of mt-Hsp70 would be required to interact with precursor proteins. Indeed, the binding of mt-Hsp70 to translocating precursor was strongly impaired in *mge1* mutant mitochondria at restrictive temperature. This interpretation is in accordance with the model for the DnaK substrate binding cycle postulated by Szabo *et al.* (1994). This model predicts that Hsp70 in the ATP-bound form is the substrate binding-competent species.

Further along these lines, GrpE proteins are known to be required for the functional nucleotide cycle of DnaK by releasing ADP or ATP from DnaK (Liberek *et al.*, 1991; Szabo *et al.*, 1994). Both unfolded protein and DnaJ stimulate the hydrolysis of ATP bound to Hsp70 and thereby increase its affinity towards already bound substrate. It is generally assumed that ADP has to be exchanged for ATP to facilitate substrate release and to allow substrate rebinding (Szabo *et al.*, 1994; for review see Hightower *et al.*, 1994). This recycling of Hsp70 from the ADP-bound to the ATP-bound form is very likely the physiological relevant role of GrpE proteins. Extending this assumption to the mitochondrial system, in *mge1* mutant mitochondria the equilibrium would be shifted to mt-Hsp70 in the ADP-bound form. As outlined above, the ADP-bound form of mt-Hsp70 is supposed to accumulate in an unproductive complex with MIM44, unable to participate in the translocation process.

A further major finding of this study is the requirement of Mge1p for folding of imported proteins. This result too can be explained by a reduced level of mt-Hsp70 competent for binding to those polypeptide chains which have been completely translocated into the matrix. Reversible binding of mt-Hsp70 to these polypeptides most likely has to occur in order to mediate folding and/or passage to mitochondrial Hsp60, which is required for folding of imported proteins to their native conformation (Ostermann *et al.*, 1989; Langer *et al.*, 1992). As during import, also during folding, the ATP-bound form of mt-Hsp70 would be the substrate binding-competent form. Binding of substrate and probably interaction with the mitochondrial DnaJ homologue, Mdj1p, then triggers ATP hydrolysis. The ADP-bound form of mt-Hsp70 would in turn have a higher affinity for substrate, and Mge1p-facilitated exchange of ADP versus ATP would be required for efficient substrate release. In summary, both functions of Mge1p in polypeptide translocation and in folding of newly imported precursor proteins are well explained by a role for Mge1p in regenerating competence for substrate binding of mt-Hsp70 by exchange of ADP versus ATP. The Mge1p-mediated nucleotide exchange at mt-Hsp70 appears to be independent of whether mt-Hsp70 is in a complex with MIM44 or Mdj1p.

The mt-Hsp70–Mge1p complex of mitochondria fulfills a very similar function as the DnaK–GrpE complex in bacteria. This can easily be explained on the basis of evolutionary conservation due to the endosymbiotic origin of mitochondria. Interestingly, a role for the mitochondrial DnaJ homologue, Mdj1p, in protein import has not been detected whereas such a role exists in the folding of imported proteins (Rowley *et al.*, 1994). In the light of the endosymbiotic origin of mitochondria, it seems that instead a novel component, namely MIM44, was recruited for the special function of the mt-Hsp70–Mge1p complex in translocating unfolded proteins across the mitochondrial membranes.

Materials and methods

Recombinant DNA techniques and plasmid constructions

General methods for manipulation of DNA were performed according to Sambrook *et al.* (1989). The *MGE1* gene with its regulatory sequences was obtained by amplification of genomic DNA of strain D273-10B by PCR using the primers BW12 (5′-CCCGGATCCTTTATCGACTA-CTGTC-3′) and BW14 (5′-CCCGAGCTCTGCAGATAATATTTTT-CTGCTC-3′). The resulting DNA fragment was subcloned into the *Bam*HI and *Sst*I sites of vector pGEM3 (Promega) yielding plasmid pBWM17. To obtain the construct for gene disruption (pBWM20), the *Sna*BI–*Bsm*I fragment of pBWM17 was replaced by the *HIS3* gene, thereby 223 bp of the promoter and 397 bp of the open reading frame of *MGE1* were removed. For expression in yeast, the *Bam*HI–*Sst*I fragment of pBWM17 was subcloned into the multicopy vector pRS426 (Christianson *et al.*, 1992) containing the *URA3* marker (pBWM23), and into the CEN vector pRS315 (Sikorski and Hieter, 1989) containing the *LEU2* marker (pBWM21).

Yeast strains and screening for temperature-sensitive mge1 mutants

Standard genetic techniques were used for growth and manipulation of yeast as described by Rose *et al.* (1990) and Sikorski and Boeke (1991). Transformation of yeast was carried out as described by Gietz *et al.* (1992).

The parental strain for construction of temperature-sensitive mutants of *MGE1* was W303a (*MAT***a**, *his3, leu2, trp1, ura3, ade2-1, can1-100*). To disrupt the genomic copy of *MGE1*, W303a was transformed with pBWM23, then the resulting strain (which is polyploid for *MGE1*) was transformed with the *Xba*I–*Sst*I fragment of pBWM20. The integration of the disrupting fragment into the genomic *MGE1* locus was confirmed by Southern blot analysis. The resulting strain, carrying a disrupted genomic copy of *MGE1* and the wild-type copy on a multicopy plasmid with the *URA3* gene as a counterselectable auxotrophic marker, was used as a recipient for mutagenized *MGE1* DNA in a plasmid shuffling experiment. After hydroxylamine mutagenesis (Rose *et al.*, 1990) pBWM21 was transformed into this strain, and after counterselection against pBWM23 on 5-fluoroorotic acid (Boeke *et al.*, 1984), 3000 transformants were screened for conditional growth by replica plating on YPD at 24 and 38°C. DNA was isolated from the ts-transformants and introduced into *E.coli* for amplification. The plasmids obtained were used for retransformation into the original recipient in order to confirm in a second plasmid shuffling experiment that the ts-phenotype was plasmid-linked. The wild-type strain used in this study was obtained by the same plasmid shuffling procedure using non-mutagenized pBWM21. Mutant and wild-type alleles were sequenced with Sequenase (United States Biochemical Corporation) according to the manufacturer's protocol using internal primers.

Synthesis of radiolabelled proteins and import in vitro

Radiolabelled precursor proteins were synthesized in reticulocyte lysate (Promega) as described (Söllner *et al.*, 1991).

Cell cultures were grown in lactate medium at 25°C and isolation of mitochondria was performed according to Daum *et al.* (1982). Import of precursor protein into isolated mitochondria was essentially carried out as described by Schwarz *et al.* (1993), with the exception that 10 mM phosphocreatine and 100 μg/ml creatine kinase were added as an ATP-regenerating system. Before addition of radiolabelled precursor protein, mitochondria were pre-incubated for 15 min at the respective temperature. For import in the presence of methothrexate (MTX), precursor protein was incubated in import buffer with 2 μM MTX, 1 mM NADPH for 10 min on ice and then warmed for 5 min at the respective temperature before it was added to an equal volume of pre-incubated mitochondria. MTX and NADPH were present during all subsequent washing steps. To deplete matrix ATP, mitochondria were incubated in import buffer without ATP and NADH for 3 min at the respective temperature, then 20 μM oligomycin and 40 U/ml apyrase were added. After a further 3 min, 5 mM NADH was added, and after an additional 9 min the import reaction was started by the addition of precursor protein. Import reactions were stopped by dilution into 4 vol ice-cold SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH, pH 7.2) containing 1 μM valinomycin. Co-immunoprecipitation of imported protein was performed as described previously (Wagner *et al.*, 1994). After SDS–PAGE and fluorography, data were quantified by laser densitometry (Ultroscan XL, Pharmacia).

Folding assay of mature Su9–DHFR

After import of pSu9(1–79)–DHFR as described above, non-imported precursor protein was digested with 100 μg/ml trypsin for 20 min on ice. Following protease inactivation by 1 mg/ml soybean trypsin inhibitor, mitochondria were re-isolated by centrifugation, resuspended to a final protein concentration of 0.5 mg/ml and then lysed in SMKCl (250 mM sucrose, 10 mM MOPS–KOH, 100 mM KCl, pH 7.2) containing 0.5% Triton X-100. After 15 min on ice, the samples were centrifuged at 16 000 *g* for 10 min at 2°C. Half of both the supernatant and the pellet were treated with 10 μg/ml proteinase K for 10 min on ice. After addition of 1 mM PMSF, trichloracetic acid precipitates were analyzed by SDS–PAGE, fluorography and densitometry. The data for the protease-treated mature Su9(1–79)–DHFR were corrected for one methionine in the Su9 moiety which becomes degraded by proteinase K.

Covalent coupling of antibodies to protein A–Sepharose

Protein A–Sepharose (200 μl wet volume; Pharmacia LKB Biotechnology, Piscataway, NJ), 500 μl 100 mM potassium phosphate buffer, pH 7.4 and 500 μl mt-Hsp70 antiserum were gently shaken for 1 h at 4°C. After two washes with 1 ml 0.2 M sodium borate buffer, pH 9.0, the protein A–Sepharose was resuspended in 1 ml sodium borate buffer, and 10 mg solid dimethyl pimelimidate (Sigma Chem. Co.) was added. After an incubation for 30 min, the coupling reaction was stopped by washing and incubation for 2 h in 0.2 M ethanolamine, pH 8.0. All steps were performed at room temperature. The Sepharose matrix with coupled antibodies was stored in TBS buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4) at 4°C. Before use, non-covalently bound antibodies were removed by washing the Sepharose matrix with 1 ml 0.1 M glycine, 0.5% Triton X-100, pH 2.5. The pH was then neutralized by four washes with 1 ml of lysis buffer (30 mM Tris, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, pH 7.4).

Co-immunoprecipitation and immunoblotting

Mitochondria (125 μg) were incubated in 250 μl import buffer at 0 or 37°C for 15 min. Re-isolated mitochondria were lysed in lysis buffer (30 mM Tris, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, pH 7.4) containing 1 mM PMSF and 0.1 mg/ml α_2 macroglobulin. When indicated in the figures, 1 mM EDTA or 3 μM to 1 mM ATP were added to the lysis buffer. After 10 min on ice, the samples were centrifuged for 15 min at 26 000 *g* at 4°C. Mt-Hsp70 antibodies pre-bound to protein A–Sepharose were added to the supernatants and the samples were kept for 1 h at 4°C with shaking. Subsequently the protein A–Sepharose was washed twice with lysis buffer, once with lysis buffer without Triton X-100 and once with 30 mM Tris, pH 7.4. Co-immunoprecipitated MIM44 and Mge1p were analysed by SDS–PAGE and immunoblotting as described (Rowley *et al.*, 1994).

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