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Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria

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ATP dependent proteolytic degradation of misfolded proteins in the mitochondrial matrix is mediated by the PIM1 protease and depends on the molecular chaperone proteins mt-hsp70 and Mdj1p. Chaperone function is essential to maintain misfolded proteins in a soluble state, a prerequisite for their degradation by PIM1 protease. In the absence of functional mt-hsp70 or Mdj1p misfolded proteins either remain associated with mt-hsp70 or form aggregates and thereby are no longer substrates for PIM1 protease. Mdj1p is shown to regulate the ATP dependent association of an unfolded polypeptide chain with mt-hsp70 affecting binding to as well as release from mt-hsp70. These findings establish a central role of molecular chaperone proteins in the degradation of misfolded proteins by PIM1 protease and thereby demonstrate a functional inter-relation between components of the folding machinery and the proteolytic system within mitochondria.

Key words: hsp70/mitochondria/molecular chaperone/protease/protein degradation

Introduction

Physiological stress, such as increased temperature, often results in denaturation of proteins within a cell. The expression of heat shock proteins is induced under these conditions to diminish damage to the cell (Lindquist and Craig, 1988; Ang *et al.*, 1991). Heat shock proteins comprise several highly conserved, but structurally unrelated protein families, which act in various manners: as molecular chaperones, some heat shock proteins stabilize folding intermediates prone to aggregation and ensure efficient refolding to the native structure under normal conditions (Pelham, 1986; Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Ellis, 1994). As proteins exhibiting proteolytic activity, other heat shock proteins are thought to degrade misfolded proteins whose renaturation cannot be achieved by molecular chaperones (Gottesman and Maurizi, 1992; Craig *et al.*, 1993). Whether proteolysis can occur after aggregation of irreversibly denatured proteins or whether a soluble state is required for degradation, is an open question. If the latter is the case, efficient proteolysis may depend also on the function of chaperone proteins. Therefore, it is conceivable that molecular chaperones and heat inducible proteases act in a coordinated manner to rid the cell of proteins that are irreversibly damaged under stress conditions.

Hsp70 proteins are present in various cellular subcompartments and are known to maintain polypeptide chains in a partially unfolded conformation thus mediating processes as complex as membrane translocation and folding of polypeptide chains, and their assembly into oligomeric structures (Dice,1990; Gething and Sambrook, 1992; Craig *et al.*, 1993; Hendrick and Hartl, 1993). In prokaryotes and eukaryotes the ATP dependent interaction of hsp70 proteins with unfolded polypeptide chains is modulated by DnaJ-like proteins which themselves exhibit chaperone function (Cyr *et al.*, 1994).

Mitochondria contain an hsp70 protein (mt-hsp70; Craig *et al.*, 1989) which is directly involved in the translocation of precursor proteins into the mitochondrial matrix and their subsequent folding (Kang *et al.*, 1990; Neupert *et al.*, 1990; Scherer *et al.*, 1990; Gambill *et al.*, 1993; Stuart *et al.*, 1994; Langer and Neupert, 1994). Recently, *MDJ1*, which encodes a mitochondrial member of the DnaJ family of proteins was identified in *Saccharomyces cerevisiae* (Rowley *et al.*, 1994). Surprisingly, deletion of *MDJ1* did not affect protein import into mitochondria, a process mediated by mt-hsp70. However, Mdj1p is required for the formation of respiratory competent mitochondria and, as is mt-hsp70, for folding of newly imported proteins in the mitochondrial matrix (Rowley *et al.*, 1994).

In contrast to the well recognized functions of mt-hsp70 in mitochondrial biogenesis, the proteolytic system of mitochondria is poorly understood. An ATP dependent protease was described in the matrix of mammalian and yeast mitochondria (Desautels and Goldberg, 1982; Watabe and Kimura, 1985; Kutejova *et al.*, 1993) and recently shown to be homologous to protease La of *Escherichia coli* (Wang *et al.*, 1993; Amerik *et al.*, 1994; Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). Disruption of the yeast *PIM1* gene, which codes for the yeast homologue of protease La, results in the formation of respiratory deficient mitochondria (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994). As with the prokaryotic homologue, expression of yeast PIM1 protease is induced upon heat shock, suggesting that the degradation of misfolded proteins is mediated by PIM1 protease. The question arises as to whether mitochondrial heat shock proteins with chaperone function, like mt-hsp70, participate in the proteolytic breakdown of proteins misfolded during heat shock.

To evaluate a possible role of mitochondrial chaperone proteins in proteolysis of misfolded proteins, we studied the degradation of newly imported, misfolded proteins. As model proteins we used a hybrid protein containing the first 167 amino acids of cytochrome b_2 fused to cytosolic mouse DHFR, which is missorted to the matrix

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as a result of specific point mutations in the presequence [$b_2(167)^{RIC}$-DHFR; Schwarz *et al.*, 1993], and bovine α-lactalbumin, which was fused to a mitochondrial presequence allowing its import into isolated yeast mitochondria. ATP dependent degradation of both proteins was observed and shown to be mediated by the PIM1 protease. Mt-hsp70 and Mdj1p were required for the proteolytic breakdown identifying them as essential components of the proteolytic machinery within the mitochondrial matrix. By preventing the aggregation of misfolded proteins mt-hsp70 and Mdj1p allow efficient proteolysis by PIM1 protease. Mdj1p ensures efficient binding of polypeptide chains to and promotes their release from mt-hsp70, a prerequisite for their degradation, thus demonstrating the cooperation of both mitochondrial chaperone proteins in this process. Taken together, these results show the functional interaction of the mitochondrial chaperone proteins mt-hsp70 and Mdj1p with the ATP dependent PIM1 protease in the proteolysis of misfolded proteins within the mitochondrial matrix.

Results

ATP dependent degradation of misfolded proteins in the mitochondrial matrix

To examine the role of mitochondrial chaperone proteins in protein degradation we developed an assay system which enabled us to distinguish chaperone function during protein import from a potential function in degradation of misfolded proteins. A chimeric protein, consisting of the first 167 amino acid residues of cytochrome b_2 and mouse DHFR, can be imported into isolated yeast mitochondria and is targeted by its bipartite presequence to the intermembrane space (Glick *et al.*, 1992; Koll *et al.*, 1992). Upon import, the first part of the bipartite presequence is removed by the mitochondrial processing peptidase in the matrix and an intermediate form (*i*-form) is generated. The intermediate form is directed by the sorting sequence to the intermembrane space, where processing to the mature form occurs. In a mutant form of this fusion protein [$b_2(167)^{RIC}$-DHFR] the amino acid residues at position 48 and 49, arginine and lysine, which are present in the wild-type cytochrome b_2 sorting sequence, were exchanged by site directed mutagenesis to isoleucine and cysteine, respectively (Schwarz *et al.*, 1993). This resulted in sorting of the protein to the matrix space instead of into the intermembrane space (Schwarz *et al.*, 1993). Upon import into the mitochondrial matrix, $b_2(167)^{RIC}$-DHFR was processed by the mitochondrial processing peptidases to the *i*-form and, in addition, to a slightly smaller form *i**. These imported forms in the matrix were degraded in a time dependent fashion, eventually leading to the formation of a fragment of ~23 kDa (*f*; Schwarz *et al.*, 1993).

In order to analyse the role of mitochondrial chaperone proteins in this degradation process the fusion protein was imported at low temperature into isolated yeast mitochondria. Complete translocation across both mitochondrial membranes occured under these conditions (data not shown), but the formation of the 23 kDa fragment *f* was almost completely blocked upon import at 15°C (Figure 1A, lane 2). After a temperature increase to 30°C, intermediate sized forms were efficiently degraded to the stable fragment *f* with a half time of ~20 min. The fragment *f* comprises the DHFR domain which is folded into the native structure prior to the temperature upshift and therefore not degraded within mitochondria. This was shown as follows: After import of $b_2(167)^{RIC}$-DHFR at 15°C mitochondria were lysed in the presence of proteinase K. Under these conditions intermediate sized forms were degraded and a fragment was formed which reacted with DHFR specific antibodies (Figure 1A; lane 1; *f*'). Thus, after import into mitochondria the DHFR domain became folded into the protease resistant native conformation which is maintained during proteolysis (data not shown). In contrast, the cytochrome b_2 moiety of the chimeric protein cannot attain the native conformation and therefore is subsequently degraded by a proteolytic system within the mitochondrial matrix. The final degradation product within mitochondria is somewhat larger than the folded DHFR domain (20.5 kDa; Figure 1A). A sterical hindrance by the folded DHFR domain or a certain sequence specificity of the mitochondrial protease may be responsible for the incomplete removal of the cytochrome b_2 moiety.

For further characterization of the proteolytic activity in the mitochondrial matrix we tested the energy requirement of the degradation process. After import of $b_2(167)^{RIC}$-DHFR either the ATP levels within mitochondria were reduced by adding apyrase, or an ATP regenerating system was added to maintain high ATP concentrations in the matrix during proteolysis. Fragment formation in the mitochondrial matrix depended strictly on the presence of ATP (Figure 1B). This observation points to an ATP dependence of the proteolytic process within the mitochondrial matrix.

We further examined the sensitivity of the proteolytic system towards the alkylating agent *N*-ethylmaleimide (NEM). Treatment of mitochondria with 1 mM NEM prior to import did not reduce the efficiency of the import reaction, however, neither *i** nor the fragment *f* were formed upon import (Figure 1C). Interestingly, after an additional incubation of NEM-treated mitochondria for 30 min at 30°C efficient degradation to *i** was observed whereas the proteolytic breakdown to fragment *f* was still completely inhibited (Figure 1C). This suggests that the two proteolytic steps exhibit a differential sensitivity towards the alkylating agent and that different proteases are involved. The *i*-form of $b_2(167)^{RIC}$-DHFR may be processed to the *i**-form by a MIP activity (mitochondrial intermediate peptidase) as MIP can be inhibited by NEM (Kalousek *et al.*, 1992). Indeed, a putative MIP cleavage site is present in the presequence of cytochrome b_2 eight amino acids after the cleavage site for the mitochondrial processing peptidase. On the other hand, the proteolytic breakdown of the *i**-form leading to the formation of the fragment *f* appears to be mediated by a so far unidentified, ATP dependent protease.

Degradation of misfolded proteins in the mitochondrial matrix is mediated by PIM1 protease, a homologue of E.coli protease La

The ATP dependence and NEM sensitivity of the degradation process points to the involvement of the recently identified mitochondrial PIM1 protease which is homologous to protease La from *E.coli* (Suzuki *et al.*, 1994;

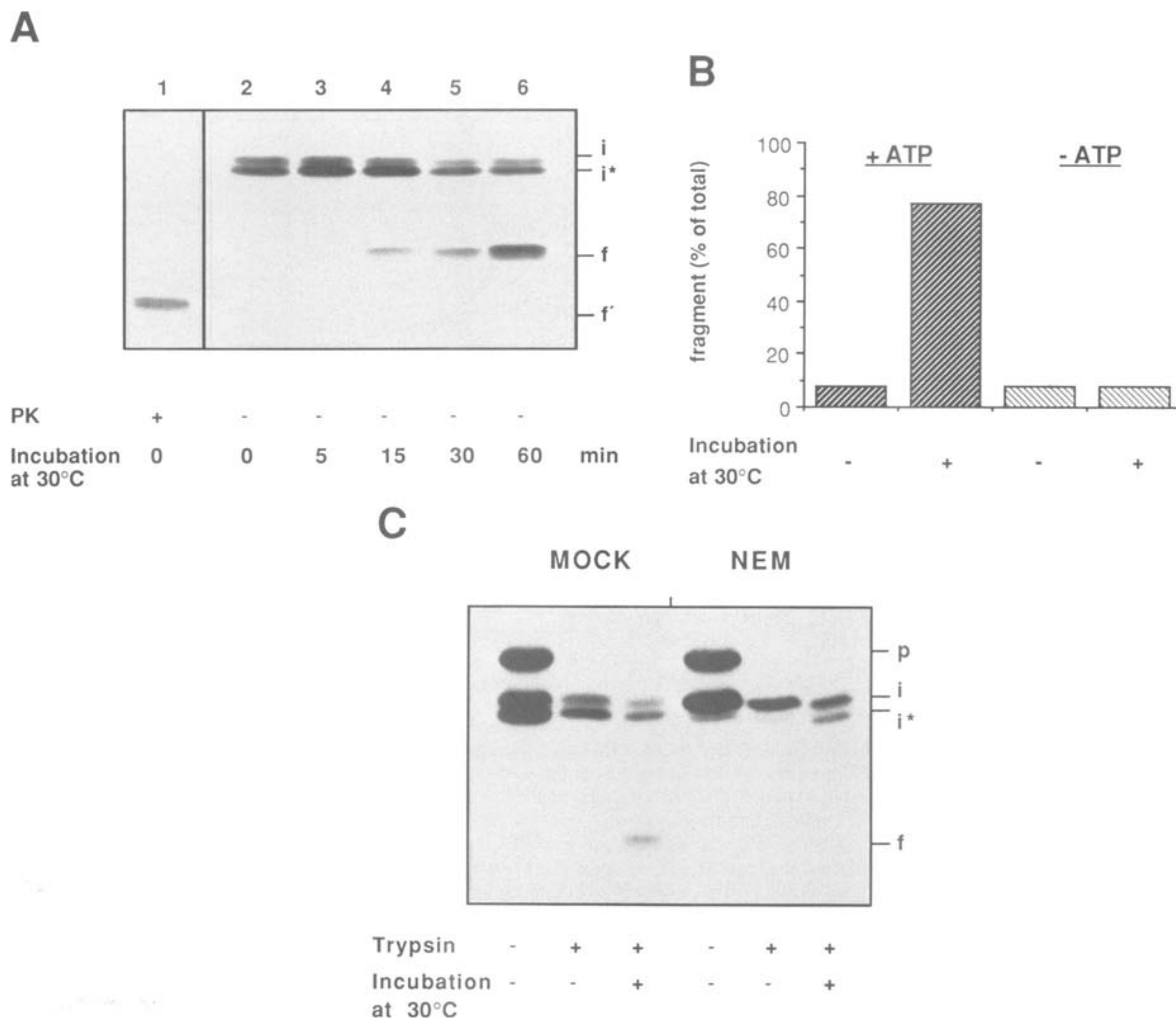

Fig. 1. Degradation of $b_2(167)^{RIC}$-DHFR missorted to the matrix space. (**A**) Kinetics of proteolytic breakdown. $b_2(167)^{RIC}$-DHFR was imported into wild-type mitochondria as described in Materials and methods. After trypsin treatment mitochondria were incubated at 30°C in import buffer (lanes 2–6). At various time points aliquots were removed and analysed by SDS–PAGE and fluorography. To assess the folding state of the DHFR moiety (lane 1) trypsin treated mitochondria were washed with SEM buffer and lysed by incubation for 30 min at 0°C in the presence of 0.5% Triton X-100 and proteinase K (50 μg/ml final concentration). Digestion was stopped by adding 2 mM PMSF. The TCA precipitated sample was analysed by SDS–PAGE and fluorography. i, i* intermediate forms of $b_2(167)^{RIC}$-DHFR; f, f' degradation products of $b_2(167)^{RIC}$-DHFR; PK, proteinase K treatment. (**B**) ATP dependence of the proteolytic breakdown of $b_2(167)^{RIC}$-DHFR. After import and trypsin treatment the sample was divided into halves. Mitochondria were reisolated by centrifugation for 10 min at 10 000 *g* and resuspended at a concentration of 0.5 mg/ml in import buffer (+ATP) or in import buffer containing apyrase (40 U/ml) and 30 μM oligomycin instead of ATP and an ATP regenerating system (–ATP). Aliquots were removed prior to and after incubation of the sample for 60 min at 30°C and analysed by SDS–PAGE. The autoradiographs were quantified by laser densitometry. (**C**) NEM sensitivity of the degradation of $b_2(167)^{RIC}$-DHFR. Mitochondria (2 mg/ml) were incubated for 10 min at 25°C in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH pH7.2) containing 1 mM NEM (NEM). In a control reaction DTT (5 mM) was also present (MOCK). To the NEM treated sample DTT (5 mM) was added and incubation continued for 5 min at 25°C. After NEM treatment mitochondria were washed with SEM buffer. $b_2(167)^{RIC}$-DHFR was imported into NEM and MOCK treated mitochondria followed by trypsin treatment to digest non-imported precursor protein as described in Materials and methods. The indicated samples were then incubated for 30 min at 30°C. Samples were analysed by SDS–PAGE and fluorography: p, precursor form of $b_2(167)^{RIC}$-DHFR; i, i*, intermediate forms of $b_2(167)^{RIC}$-DHFR; f, degradation product of $b_2(167)^{RIC}$-DHFR.

Van Dyck *et al.*, 1994). PIM1 protease is known to degrade [^{3}H]casein in an ATP dependent manner *in vitro*, an activity which can be inhibited by NEM treatment of the protease (Kutejova *et al.*, 1993). To test the participitation of PIM1 protease, $b_2(167)^{RIC}$-DHFR was imported into mitochondria that were isolated from a *PIM1* deletion strain (Van Dyck *et al.*, 1994). During import, processing of $b_2(167)^{RIC}$-DHFR to the intermediate forms *i* and *i** was observed. However, in contrast to wild type, no fragment *f* was formed in $\Delta pim1$ mutant mitochondria (Figure 2A). This shows that the PIM1 protease mediates the degradation of missorted $b_2(167)^{RIC}$-DHFR and, moreover, that under the experimental conditions used another mitochondrial protease cannot efficiently substitute for PIM1 protease function. The *PIM1* gene is not essential for the life of yeast cells, but its deletion results in loss of the mitochondrial respiratory system, thereby causing a severe disturbance of the energy metabolism. The effect of the *PIM1* deletion on the degradation of $b_2(167)^{RIC}$-DHFR, however, appears to be specific because the ATP levels were kept high by adding an ATP regenerating system. In addition, degradation of $b_2(167)^{RIC}$-DHFR in strain AB972 was observed (Figure 2A). This strain is also petite and has lost mitochondrial DNA (Rose *et al.*, 1993).

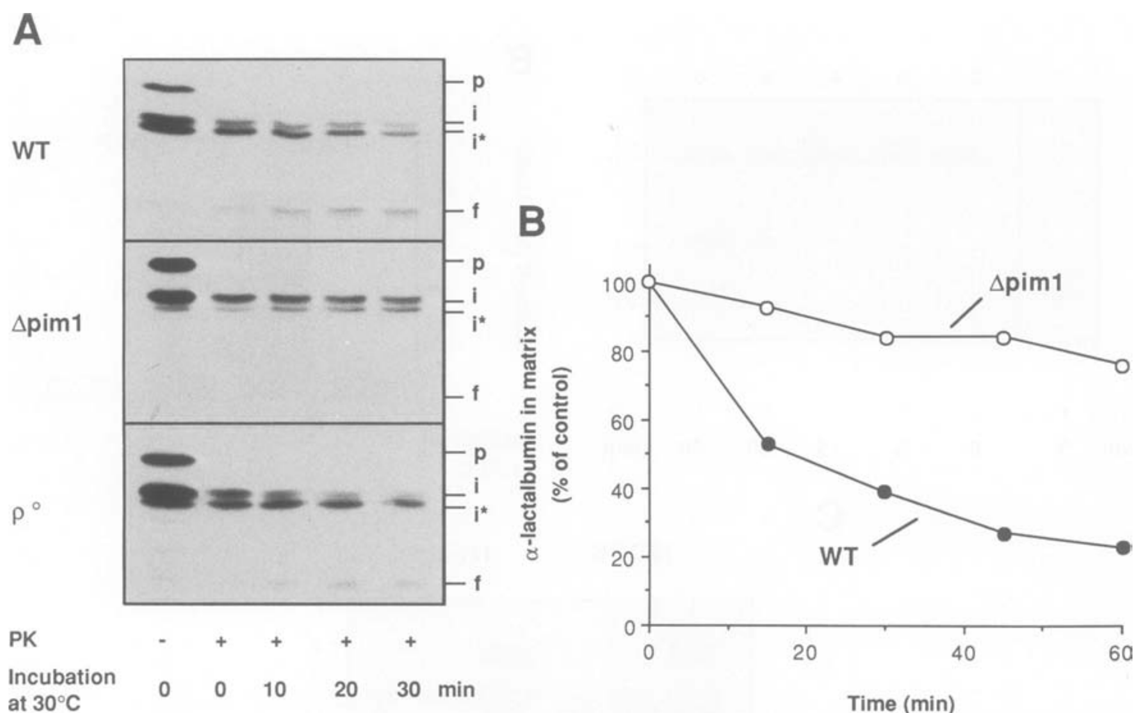

Fig. 2. Misfolded proteins in the mitochondrial matrix are degraded by the PIM1 protease. (**A**) Degradation of $b_2(167)^{RIC}$-DHFR is blocked in Δ*pim1* mitochondria. Import of $b_2(167)^{RIC}$-DHFR into mitochondria isolated from the wild-type strain FY73 (WT), the *PIM1* deletion strain (Δ*pim1*) and from AB972 (ρ^0) was carried out as described in Materials and methods. Proteinase K treated samples were incubated in import buffer at 30°C for various times as indicated and analysed by SDS–PAGE and fluorography. Abbreviations see legend to Figure 1. (**B**) Degradation of Su9(1–69)–lactalbumin is blocked in Δ*pim1* mitochondria. Su9(1–69)–lactalbumin was imported into wild-type (FY73) and Δ*pim1* mitochondria followed by a proteinase K treatment of the sample as described in Materials and methods. The samples were incubated at 30°C for various times as indicated and analysed by SDS–PAGE, fluorography and laser densitometry of the autoradiographs. Imported Su9(1–69)–lactalbumin prior to degradation was set to 100%.

We examined the function of the *PIM1* protease in degradation of another misfolded polypeptide chain in the mitochondrial matrix, bovine α-lactalbumin. Folding of this normally secreted protein to the native structure requires the formation of four disulfide bridges in the lumen of the endoplasmic reticulum. We exploited this observation to accumulate an unfolded polypeptide chain in the matrix space of mitochondria. The strong reducing environment within mitochondria can be expected to prevent the formation of disulfide bridges and consequently the folding of α-lactalbumin. The signal sequence of α-lactalbumin was replaced by an amino-terminal fragment of the precursor of the mitochondrial F_O-ATPase subunit 9, comprising the mitochondrial presequence and three amino acid residues of the mature protein. The resulting hybrid protein was efficiently imported into isolated yeast mitochondria. Degradation of α-lactalbumin in the matrix occurred with a half time of ~20 min (Figure 2B). In mitochondria isolated from the Δ*pim1* strain, the imported α-lactalbumin was degraded at a severely reduced rate, again demonstrating the importance of the mitochondrial PIM1 protease for the degradation of misfolded proteins in the mitochondrial matrix (Figure 2B).

Degradation of misfolded proteins requires functional mt-hsp70

After import into mitochondria and lysis, $b_2(167)^{RIC}$-DHFR was recovered in the soluble fraction (data not shown). We reasoned that mt-hsp70 may be required to prevent the aggregation of misfolded proteins and thus allow efficient degradation by PIM1 protease. To test this hypothesis degradation of $b_2(167)^{RIC}$-DHFR was examined in mitochondria which contain conditional mutant forms of mt-hsp70. Whereas the *ssc1-3* mutant protein was reported to be incapable of binding to a polypeptide chain during its import (Gambill *et al.*, 1993), the *ssc1-2* mutant protein could form stable complexes with proteins newly imported into mitochondria (Kang *et al.*, 1990). After import of $b_2(167)^{RIC}$-DHFR into *ssc1-3* mutant mitochondria, samples were incubated at 37°C to induce the phenotype. Whereas the increase of the temperature resulted in proteolytic breakdown of $b_2(167)^{RIC}$-DHFR in wild-type mitochondria, no fragment *f* was formed in *ssc1-3* mitochondria (Figure 3), thus demonstrating the requirement of mt-hsp70 for efficient degradation. $b_2(167)^{RIC}$-DHFR was recovered as an aggregated form, which apparently cannot be degraded by the PIM1 protease. Thus, efficient proteolysis by PIM1 protease requires a soluble state of the misfolded polypeptide chain.

We then asked whether the binding function of mt-hsp70 would be sufficient to allow proteolysis to occur. $b_2(167)^{RIC}$-DHFR was imported into *ssc1-2* mitochondria at permissive temperature. As in the *ssc1-3* mutant, fragment formation was completely blocked after shift to non-permissive temperature (Figure 3). Unfolding of the DHFR domain of the chimeric fusion protein was observed in *ssc1-2* mutant mitochondria, but not in wild-type mitochondria under these conditions (data not shown). In a similar manner, degradation of α-lactalbumin was blocked at non-permissive temperature in *ssc1-2* mitochon-

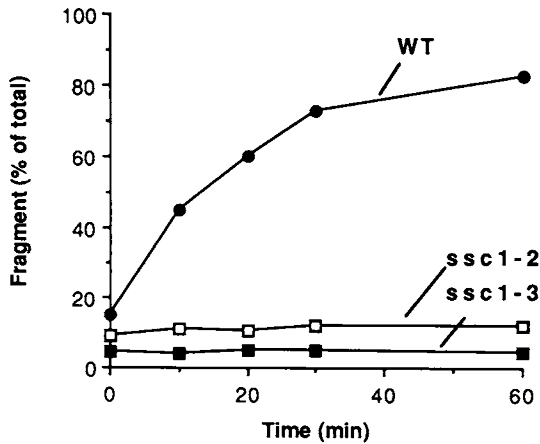

Fig. 3. Degradation of $b_2(167)^{RIC}$-DHFR requires mt-hsp70. $b_2(167)^{RIC}$-DHFR was imported into wild-type, *ssc1-2* and *ssc1-3* mutant mitochondria at permissive temperature as described in Materials and methods. Trypsin treated mitochondria were incubated at 37°C to induce the phenotype and to allow proteolysis to occur. At various time points aliquots were removed and fragment formation was quantified by SDS–PAGE, fluorography and laser densitometry of the autoradiographs.

dria (data not shown). This suggests that binding of mt-hsp70 to a misfolded protein alone does not facilitate its degradation by PIM1 protease.

Release of a misfolded protein from mt-hsp70 precedes its degradation by PIM1 protease

To investigate in more detail the role of mt-hsp70 in the degradation process, coimmunoprecipitation experiments were carried out in wild-type and in *ssc1-2* mitochondria. Using an antibody raised against purified mt-hsp70, *i*- and *i**-$b_2(167)^{RIC}$-DHFR were coimmunoprecipitated after import, demonstrating a direct physical interaction with mt-hsp70. The efficiency of the coimmunoprecipitation was ~5–10 % in wild-type and in *ssc1-2* mitochondria at permissive temperature (Figure 4A). This is comparable with earlier results (Kang *et al.*, 1990; Gambill *et al.*, 1993). Insufficient ATP depletion of the matrix probably is the reason for only partial coimmunoprecipitation. A strong increase in the efficiency of coimmunoprecipitation was observed in *ssc1-2* mitochondria after incubation for 5 min at the non-permissive temperature (Figure 4A). This may reflect an increase in the apparent affinity of the *ssc1-2* mutant protein for unfolded polypeptides and, as a consequence of the unfolding of the DHFR domain in *ssc1-2* mutant mitochondria under these conditions (data not shown), the increased number of potential binding sites for mt-hsp70. We analysed the functional defect of the *ssc1-2* mutant protein in more detail by directly assessing ATP dependent release from mt-hsp70 during the proteolytic breakdown of $b_2(167)^{RIC}$-DHFR. After import, proteolysis was started by increasing the temperature to 37°C. At various time points aliquots were taken and coimmunoprecipitation with mt-hsp70-specific antibodies was carried out (Figure 4B). In wild-type mitochondria $b_2(167)^{RIC}$-DHFR was efficiently released from mt-hsp70 with a half time of ~20 min and thus paralleled the formation of the fragment *f*. In contrast, in *ssc1-2* mitochondria *i*- and *i**-$b_2(167)^{RIC}$-DHFR remained bound during incubation at 37°C (Figure 4B).

Hsp70 proteins interact with substrate proteins in an ATP dependent manner. Therefore, removal of ATP may cause block of degradation by preventing release of $b_2(167)^{RIC}$-DHFR from mt-hsp70 rather than inhibiting the proteolytic activity of the PIM1 protease itself. To localize the block in the proteolytic breakdown of $b_2(167)^{RIC}$-DHFR in the absence of ATP, the association with mt-hsp70 was assessed at normal and at reduced matrix ATP levels. Whereas in the presence of high ATP levels release of $b_2(167)^{RIC}$-DHFR was observed, ATP depletion by adding apyrase and oligomycin resulted in accumulation of the intermediate forms at mt-hsp70 (Figure 4C). The observed increase most likely reflects the reduction of ATP levels over time. This demonstrates the presence of at least two ATP dependent steps during proteolysis of $b_2(167)^{RIC}$-DHFR: binding and release from mt-hsp70, and proteolytic breakdown of the polypeptide chain by the ATP dependent PIM1 protease.

These results establish the requirement of functional mt-hsp70 for efficient degradation of misfolded proteins by the mitochondrial PIM1 protease. As indicated by the presence of a folded, protease resistant DHFR domain prior to and during the proteolytic breakdown (Figure 1A), mt-hsp70 appears to bind specifically to the cytochrome b_2 part of the hybrid protein prone to degradation. The association of mt-hsp70 with the folding-incompetent moiety of the hybrid protein apparently prolongs its half life in a soluble conformation, preventing aggregation and allowing efficient proteolysis by the PIM1 protease. As demonstrated by the block of degradation under restrictive conditions in the *ssc1-2* mutant mitochondria, PIM1 protease cannot mediate the proteolysis of unfolded $b_2(167)^{RIC}$-DHFR associated with mt-hsp70. Rather, degradation by PIM1 protease requires release of the misfolded protein from mt-hsp70. Concomitant with the release, efficient proteolysis occurs as is demonstrated by similar kinetics of the release reaction from mt-hsp70 and of the formation of the proteolytic fragment *f*.

Misfolded proteins aggregate in the Δpim1 mutant upon release from mt-hsp70

To unravel further the role of mt-hsp70 during proteolysis we examined the fate of $b_2(167)^{RIC}$-DHFR in the Δ*pim1* strain. In the absence of PIM1 protease a misfolded protein incapable of attaining the native conformation may remain associated with mt-hsp70. Deletion of *PIM1*, however, did not significantly affect the efficiency of binding of $b_2(167)^{RIC}$-DHFR to or the release from mt-hsp70 which occurs with similar kinetics in Δ*pim1* to those in wild-type mitochondria (Figure 5A). Upon release from mt-hsp70 *i*- and *i**-$b_2(167)^{RIC}$-DHFR aggregated and were recovered in the pellet fraction of Δ*pim1* mitochondria but not in wild-type mitochondria (Figure 5B). In agreement with this finding, α-lactalbumin was also recovered in the pellet fraction of Δ*pim1* mitochondria (data not shown). These results indicate that mt-hsp70 is required to prevent the aggregation of polypeptide chains prone to degradation and to maintain them in a soluble state. Binding to and release from mt-hsp70, however, turns out to be independent from the proteolytic breakdown.

Mdj1p is required for proteolysis by modulating substrate binding by mt-hsp70

In order to analyse the effect of NEM on degradation we tested the NEM sensitivity of binding to and release from

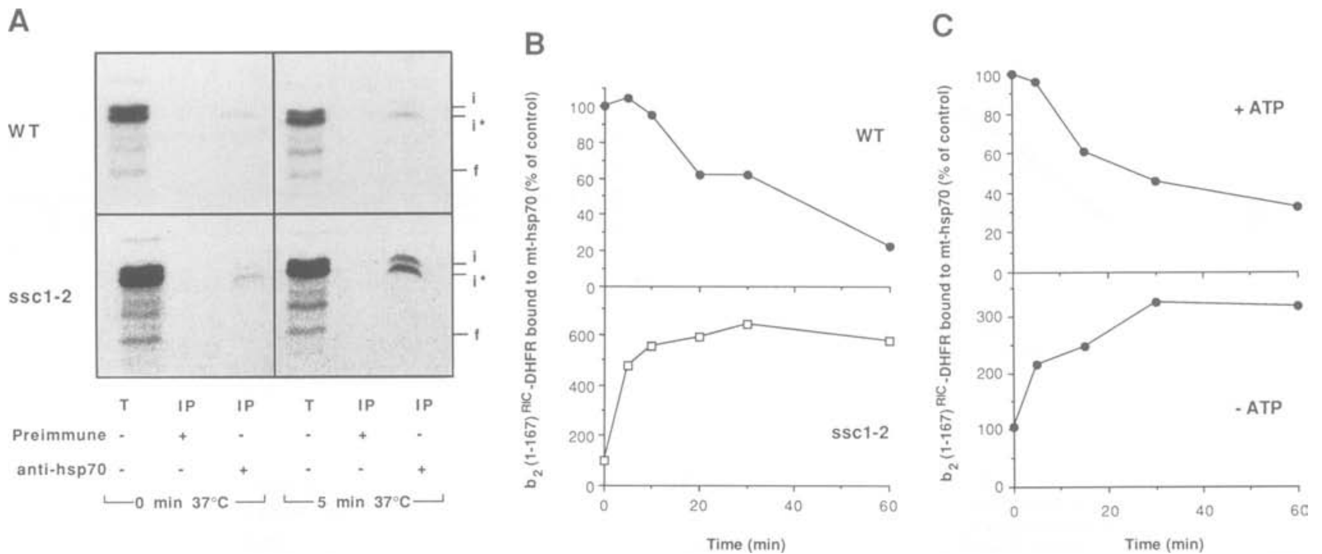

Fig. 4. Association of $b_2(167)^{RIC}$-DHFR with mt-hsp70. $b_2(167)^{RIC}$-DHFR was imported into wild-type and *ssc1-2* mutant mitochondria followed by trypsin treatment of mitochondria as described in Materials and methods. (**A**) Coimmunoprecipitation of $b_2(167)^{RIC}$-DHFR with mt-hsp70. After import, samples were divided into halves and were incubated at 0°C or 37°C. At zero time aliquots were removed for control (T). The remaining samples were kept for 5 min at 0°C or at 37°C. Association with mt-hsp70 was determined by coimmunoprecipitation (IP) as described in Materials and methods using mt-hsp70 specific antiserum and, as a control, preimmune serum. Abbreviations see legend to Figure 1. (**B**) Release from mt-hsp70 is blocked in *ssc1-2* mitochondria. After import trypsin treated mitochondria were incubated at 37°C. At the time points indicated aliquots were withdrawn and coimmunoprecipitation with hsp70-specific antiserum was carried out as described in Materials and methods. (**C**) ATP dependence of the release of $b_2(167)^{RIC}$-DHFR from mt-hsp70. After import of $b_2(167)^{RIC}$-DHFR in wild-type mitochondria and trypsin treatment the sample was divided into halves. Mitochondria were reisolated by centrifugation for 10 min at 10 000 *g* and resuspended at a concentration of 0.5 mg/ml in import buffer (+ATP) or in import buffer containing apyrase (40 U/ml) and 30 μM oligomycin, instead of ATP and an ATP regenerating system (–ATP). Then, mitochondria were incubated at 30°C. At the time points indicated coimmunoprecipitation was performed as described in Materials and methods. In (B) and (C) $b_2(167)^{RIC}$-DHFR bound to mt-hsp70 prior to degradation was set to 100 %.

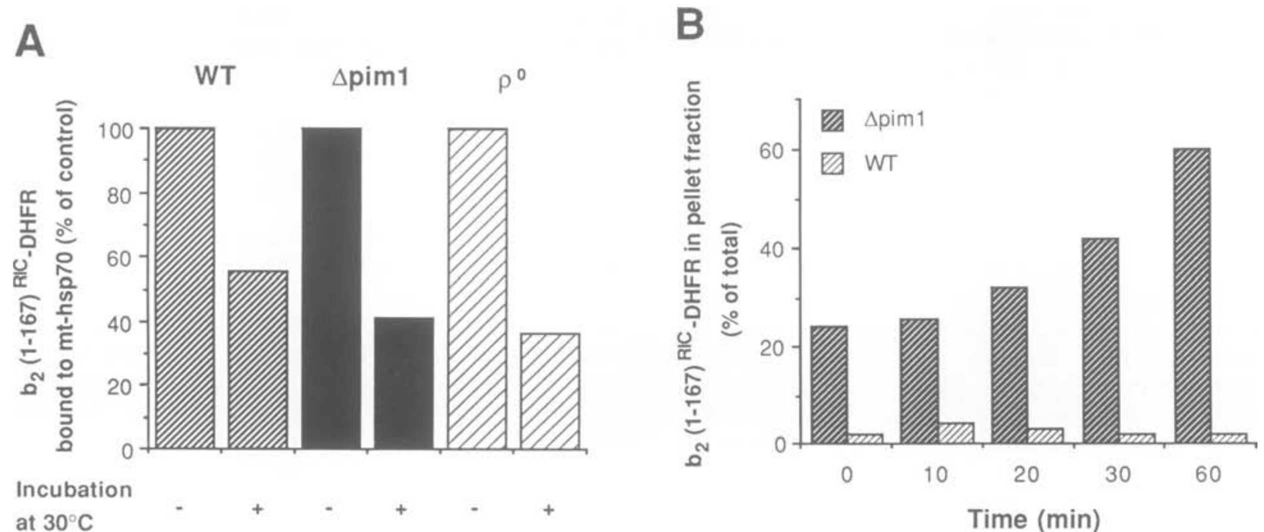

Fig. 5. Misfolded proteins aggregate after release from mt-hsp70 in *Δpim1* mutant mitochondria. (**A**) Release of $b_2(167)^{RIC}$-DHFR from mt-hsp70 is not affected in *Δpim1* mitochondria. $b_2(167)^{RIC}$-DHFR was imported into mitochondria isolated from the *PIM1* deletion strain (Δpim1), the isogenic wild-type strain (FY73; WT) and from AB972 (ρ^0). After proteinase K treatment mitochondria were incubated for 30 min at 30°C. Then mitochondria were lysed in IPP buffer and coimmunoprecipitation with hsp70 specific antiserum was carried out. Samples were analysed by SDS–PAGE and quantified by laser densitometry of the autoradiographs. $b_2(167)^{RIC}$-DHFR bound to mt-hsp70 prior to incubation at 30°C was set to 100 %. (**B**) Aggregation of $b_2(167)^{RIC}$-DHFR in *Δpim1* mutant mitochondria. After import as in (A), mitochondria were incubated at 30°C. Aliquots were removed at the time points indicated. Mitochondria were reisolated by centrifugation for 15 min at 20 000 *g* and lysed by incubation for 15 min at 0°C in IPP buffer at a concentration of 1 mg/ml. Aggregation of $b_2(167)^{RIC}$-DHFR was analysed by centrifugation for 15 min at 25 000 *g*. The pellet fraction was dissolved in SDS–PAGE sample buffer. Protein in the supernatant was precipitated with TCA. Then, both fractions were subjected to SDS–PAGE.

mt-hsp70. Mitochondria were incubated with 1 mM NEM in the presence and absence of 5 mM DTT prior to import. NEM did not affect the efficiency of import or binding to mt-hsp70, but ATP dependent release of $b_2(167)^{RIC}$-DHFR from mt-hsp70 was impaired (Figure 6A). Since mt-hsp70 from *S.cerevisiae* lacks cysteine residues (Craig *et al.*, 1989) it seems possible that another component affects the ATP dependent release from mt-hsp70. A likely

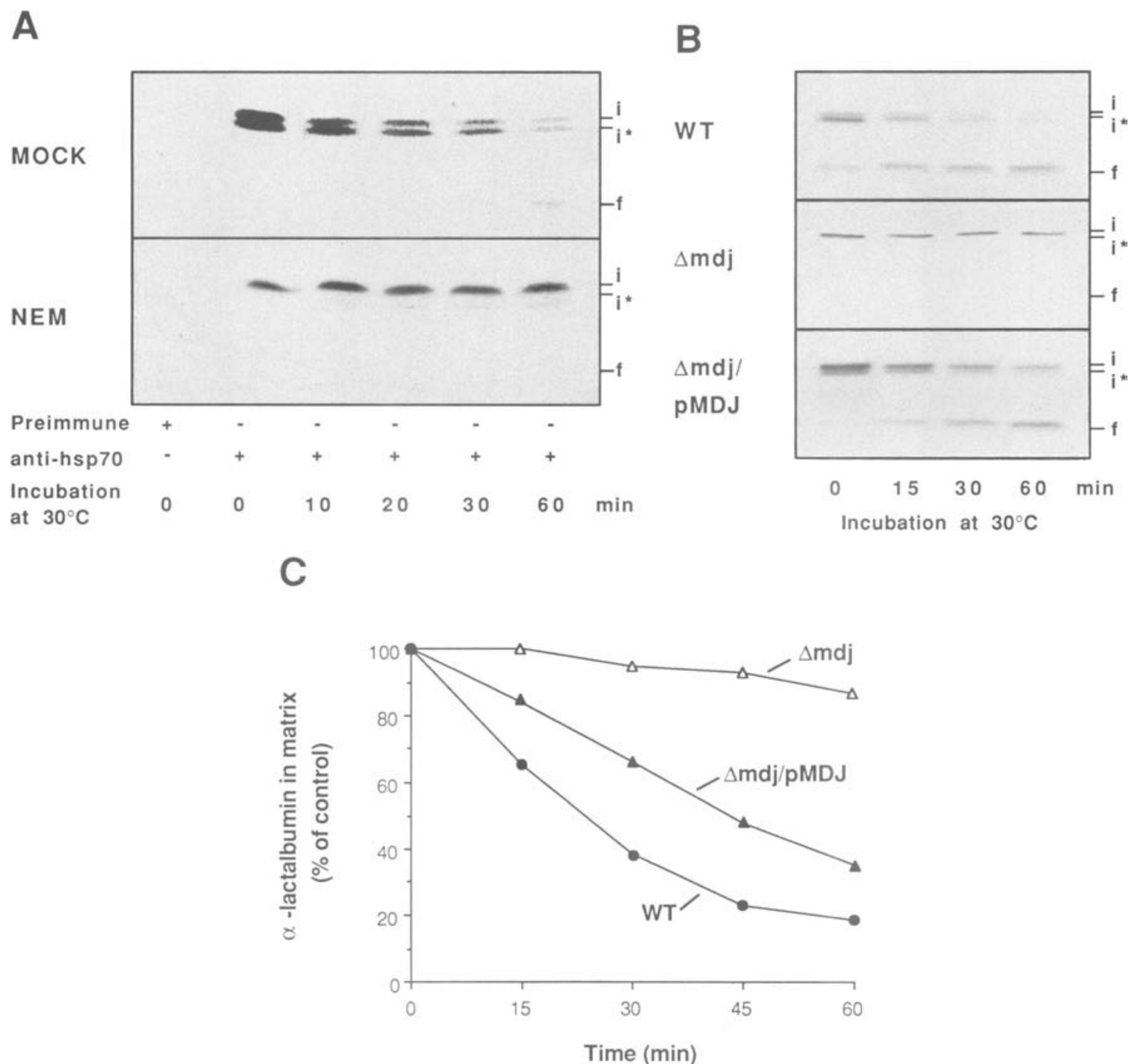

Fig. 6. Degradation of $b_2(167)^{RIC}$-DHFR is NEM sensitive and depends on Mdj1p. (**A**) NEM sensitivity of the release of $b_2(167)^{RIC}$-DHFR from mt-hsp70. Mitochondria were treated with 1 mM NEM prior to import as described in Figure 1C. After import and proteinase K treatment samples were incubated at 30°C. At the time points indicated, association with mt-hsp70 was determined by coimmunoprecipitation. Abbreviations see legend to Figure 1. (**B**) Degradation of $b_2(167)^{RIC}$-DHFR is blocked in *Δmdj1* mutant mitochondria. $b_2(167)^{RIC}$-DHFR was imported into mitochondria from the *Δmdj1* mutant strain (Δmdj1), the *Δmdj1* mutant strain supplemented with a wild-type copy of the *MDJ1* gene (Δmdj1/pMDJ1), and the isogenic wild-type strain (WT). After trypsin treatment mitochondria were incubated in import buffer at 30°C for various times as indicated. The mitochondria were reisolated and analysed by SDS–PAGE. Abbreviations see legend to Figure 1. (**C**) Degradation of α-lactalbumin is blocked in *Δmdj1* mutant mitochondria. Import of Su9 (1–69)–lactalbumin into mitochondria was carried out as described in Materials and methods. After digestion of non-imported precursor protein by proteinase K mitochondria were incubated at 30°C. At the time points indicated, mitochondria were reisolated. Degradation of α-lactalbumin was analysed by SDS–PAGE and quantified by laser densitometry of the autoradiographs. α-lactalbumin in the matrix prior to incubation at 30°C was set to 100%.

candidate is Mdj1p which was recently shown to be involved in folding of mitochondrial proteins in the matrix space (Rowley *et al.*, 1994). Although a functional interaction with mt-hsp70 has not been demonstrated, it is likely to occur since Mdj1p belongs to the family of DnaJ-like proteins known to modulate the ATP dependent interaction of hsp70 proteins with unfolded polypeptide chains (Cyr *et al.*, 1994).

To examine the role of Mdj1p in proteolysis of misfolded mitochondrial proteins we analysed the degradation of $b_2(167)^{RIC}$-DHFR in a yeast strain which harbours a disrupted *MDJ1* gene. As shown in Figure 6B, the proteolytic fragment *f* was not formed in mitochondria isolated from the *Δmdj* yeast strain. After expression of Mdj1p from a single copy plasmid in the *Δmdj* strain, degradation of $b_2(167)^{RIC}$-DHFR was observed, suggesting a direct role of Mdj1p in proteolysis (Figure 6B). In a similar manner, degradation of α-lactalbumin was blocked in the absence of Mdj1p (Figure 6C), indicating a general function of Mdj1p in the degradation of misfolded proteins.

If Mdj1p interacts with mt-hsp70, binding of $b_2(167)^{RIC}$-DHFR to mt-hsp70 may be affected in *Δmdj* mutant mitochondria. Therefore, coimmunoprecipitation experiments with hsp70 specific antibodies were carried out during proteolytic breakdown of $b_2(167)^{RIC}$-DHFR. After import, binding to mt-hsp70 was strongly reduced in *Δmdj* mitochondria (Figure 7A). This reduction of binding to mt-hsp70 was paralleled by aggregation of $b_2(167)^{RIC}$-DHFR (Figure 7B). In addition, Mdj1p was required for the ATP dependent release of $b_2(167)^{RIC}$-DHFR from

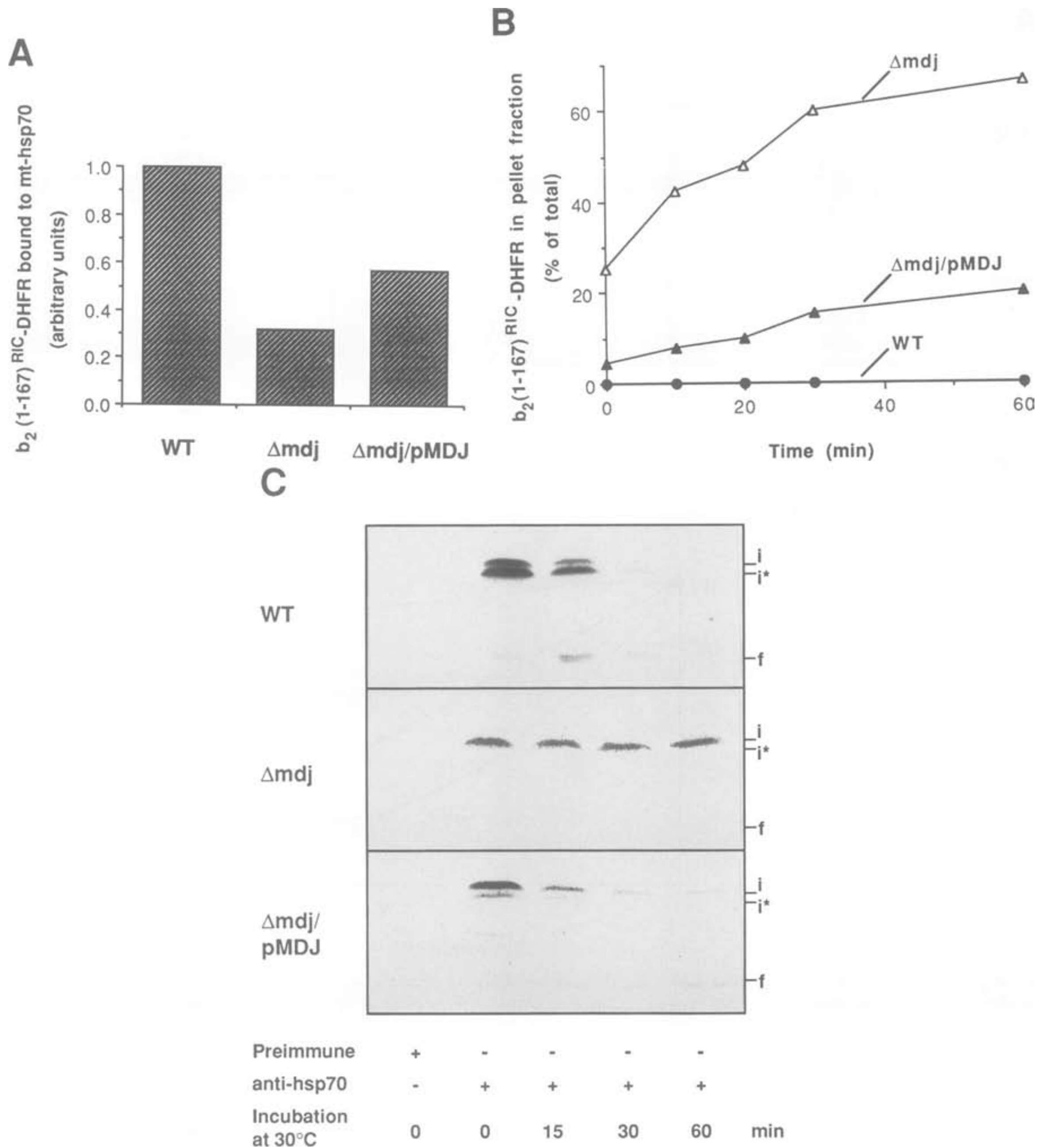

Fig. 7. Mdj1p regulates substrate binding by mt-hsp70. (**A**) Binding of $b_2(167)^{RIC}$-DHFR is decreased in *Δmdj1* mitochondria. $b_2(167)^{RIC}$-DHFR was imported into mitochondria from the *Δmdj1* mutant strain (Δmdj1), the *Δmdj1* mutant strain supplemented with a wild-type copy of the *MDJ1* gene (Δmdj1/pMDJ1), and the isogenic wildtype strain (WT). Binding of $b_2(167)^{RIC}$-DHFR to mt-hsp70 was analysed by coimmunoprecipitation as described in Materials and methods followed by SDS–PAGE and laser densitometry of the autoradiographs. Binding to mt-hsp70 was corrected for the efficiency of import into mitochondria isolated from different strains. $b_2(167)^{RIC}$-DHFR bound to mt-hsp70 in wild-type mitochondria was set to 1.0. (**B**) Aggregation of $b_2(167)^{RIC}$-DHFR in *Δmdj1* mutant mitochondria. After import of $b_2(167)^{RIC}$-DHFR as in (A), mitochondria were incubated at 30°C. At the time points indicated, aliquots were withdrawn and aggregation of $b_2(167)^{RIC}$-DHFR was determined as described in Figure 5(B). (**C**) Release of $b_2(167)^{RIC}$-DHFR is blocked in *Δmdj1* mutant mitochondria. After import of $b_2(167)^{RIC}$-DHFR as in (A), trypsin treated mitochondria were incubated at 30°C. At time points indicated, binding of $b_2(167)^{RIC}$-DHFR to mt-hsp70 was determined by coimmunoprecipitation as described in Materials and methods using mt-hsp70-specific antiserum and, as a control, preimmune serum. Abbreviations see legend to Figure 1.

mt-hsp70. $b_2(167)^{RIC}$-DHFR remained bound stably to mt-hsp70 in the absence of Mdj1p. In contrast, mt-hsp70 associated protein was released with similar kinetics in wild type mitochondria and in mitochondria from a *Δmdj* strain supplemented with plasmid expressed Mdj1p (Figure 7C). These results demonstrate the requirement of Mdj1p for degradation of misfolded proteins in the mitochondrial matrix. Mdj1p functionally interacts with mt-hsp70 in this process, affecting the binding of a misfolded protein to, as well as its release from, mt-hsp70.

Discussion

We characterized a proteolytic system in the mitochondrial matrix which promotes the degradation of misfolded proteins in an ATP dependent manner. PIM1 protease, a

homologue of the *E.coli* protease La, as well as the mitochondrial chaperone proteins mt-hsp70 and Mdj1p were identified as essential components of this degradation pathway thereby demonstrating their functional interaction during proteolysis within mitochondria.

PIM1 protease, previously known to degrade ^{3}H-labelled casein *in vitro* and be involved in the regulated turnover of certain mitochondrial proteins (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994), mediates the ATP dependent proteolytic breakdown of misfolded proteins in the mitochondrial matrix. In mitochondria lacking PIM1 protease the degradation rate of misfolded model proteins was dramatically reduced and aggregates of the proteins were formed. Thus, other putative mitochondrial proteases cannot efficiently substitute for the loss of PIM1 protease. In a similar manner, the *E.coli* homologue to PIM1 protease, the La protease (Chin *et al.*, 1988), mediates the ATP dependent degradation of misfolded proteins (Bukhari and Zipser, 1973; Waxman and Goldberg, 1986) and certain regulatory proteins (Gottesman *et al.*, 1981; Mizusawa and Gottesman, 1983; Stout *et al.*, 1991). In *E.coli*, proteases with substrate specificities similar to protease La appear to exist, one of which might be the ATP dependent Clp protease (Maurizi *et al.*, 1985; Hwang *et al.*, 1987). A homologue of the regulating subunit of the *E.coli* Clp protease was recently identified in the mitochondrial matrix and denoted as hsp78 (Leonhardt *et al.*, 1993). However, hsp78 does not appear to promote the degradation of misfolded proteins in the mitochondrial matrix in the absence of PIM1 protease under the conditions tested.

Misfolded proteins, which exhibit a strong tendency to aggregate, are recognized only by the PIM1 protease if they are present in a soluble conformation. Molecular chaperone proteins fulfill an essential function during proteolysis by preventing the aggregation of misfolded proteins, thereby allowing their degradation by PIM1 protease. The requirement of functional mt-hsp70 for efficient proteolysis is demonstrated by the complete block of degradation at non-permissive temperature in the conditional mutants *ssc1-2* and *ssc1-3*, in which misfolded proteins do not dissociate from mt-hsp70 or form aggregates, respectively. These results are in agreement with earlier observations which suggested a role of hsp70 proteins in proteolytic processes in *E.coli*. DnaK, the prokaryotic homologue of the hsp70 class of heat shock proteins, appears to be involved in the proteolysis of misfolded proteins and puromycyl fragments, as this process was affected in *DnaK* deletion strains (Keller and Simon, 1988; Straus *et al.*, 1988; Sherman and Goldberg, 1992). Therefore, the function of hsp70 proteins in proteolytic breakdown of misfolded proteins appears to be conserved in prokaryotes and eukaryotes.

The study of the degradation of misfolded proteins in the mitochondrial matrix also provided insights into the functional interaction of hsp70 proteins with DnaJ-like proteins, because, in addition to mt-hsp70, Mdj1p is required for efficient proteolysis. Mdj1p belongs to the family of DnaJ-like proteins which can act as molecular chaperones and are known to modulate the ATP dependent interaction of hsp70 proteins with unfolded polypeptide chains (Cyr *et al.*, 1994). Mdj1p was shown to be involved in the folding of newly imported proteins in the matrix space of mitochondria (Rowley *et al.*, 1994). Our findings establish a crucial role of Mdj1p during proteolysis and its functional interaction with mt-hsp70 during this process. Mdj1p affects the binding of a misfolded protein to, as well as the release from, mt-hsp70. The observed reduced binding efficiency of mt-hsp70 to an unfolded polypeptide chain in the absence of Mdj1p is in agreement with *in vitro* studies using the purified *E.coli* homologues DnaK and DnaJ. These studies show an increased affinity of DnaK for various substrate proteins in the presence of DnaJ (Wickner *et al.*, 1991; Langer *et al.*, 1992; Osipiuk *et al.*, 1993; Schröder *et al.*, 1993). Mdj1p may act as a molecular chaperone and bind directly to the polypeptide chain prone to degradation thereby allowing more efficient binding of mt-hsp70. Alternatively, direct physical interaction of Mdj1p with the substrate protein may not occur. Rather a high affinity conformation of mt-hsp70 may be stabilized by the direct interaction between Mdj1p and mt-hsp70.

Surprisingly, release of an unfolded protein from mt-hsp70 was blocked in the absence of Mdj1p. Stimulation of the ATPase activity of mt-hsp70 by Mdj1p may be required to obtain substrate release. In a similar manner, the yeast cytosolic DnaJ homologue Ydj1p promotes the release of reduced carboxymethylated α-lactalbumin (R-CMLA) from Ssa1p *in vitro* (Cyr *et al.*, 1992). R-CMLA lacks secondary structure elements and is not bound by Ydj1p. However, it appears that DnaJ-like proteins affect the stability of hsp70–substrate complexes in a manner dependent on the conformation of the substrate protein. If polypeptide chains exhibit structural elements recognized by DnaJ-like proteins, they are not released from hsp70 under otherwise identical conditions (Langer *et al.*, 1992; Hoffmann *et al.*, 1992; Cyr *et al.*, 1994). It remains to be determined whether mitochondrial proteins prone to degradation are bound by Mdj1p during the proteolytic breakdown and whether deletion of *MDJ1* results in a block of the ATP dependent release of such proteins from mt-hsp70.

ATP dependent release at least of certain misfolded proteins from mt-hsp70 depends on Mdj1p and, most likely, on the recently identified mitochondrial GrpE homologue Mge1p (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Laloraya *et al.*, 1994). In mitochondria isolated from a Mdj1p deficient strain and in NEM treated mitochondria the tested substrate protein remained bound to mt-hsp70. As cysteine residues are lacking in mt-hsp70 and Mge1p, Mdj1p appears to be the major target of NEM. If this is the case, Mdj1p is partially functional after NEM treatment, as under these conditions, in contrast to what was observed when the *MDJ1* gene was deleted, binding of misfolded proteins was not affected. The NEM sensitivity of the release of unfolded proteins from mt-hsp70 is reminiscent of the observation that dissociation of precursor proteins from cytosolic hsp70, prior to translocation into the endoplasmic reticulum, requires a so far unidentified, NEM sensitive factor (Murakami *et al.*, 1988; Chirico, 1992). Further studies should reveal a possible relationship of both NEM sensitive processes.

Is the interaction of molecular chaperone proteins with the proteolytic system in the mitochondrial matrix regulated? PIM1 protease does not mediate the degradation of misfolded proteins still associated with mt-hsp70. Rather, release of mt-hsp70 is required for efficient proteolysis.

On the other hand, this observation does not exclude the possibility that the PIM1 protease binds to a polypeptide chain still associated with mt-hsp70 or Mdj1p. The observed binding of the degradation fragment *f* of $b_2(167)^{RIC}$-DHFR to mt-hsp70 (Figure 6A and 7C) suggests that proteolysis occurs in cycles of binding to and release from mt-hsp70. Maintenance of an unfolded conformation by these mitochondrial chaperones may enable the PIM1 protease to efficiently bind misfolded proteins and, after release of the chaperone proteins, promote their degradation. Interestingly, we have observed that deletion of the *PIM1* gene decreases the solubility of a model protein in NEM treated mitochondria (I.Wagner, T.Langer and W.Neupert, unpublished observation). This puzzling observation might be explained by binding of PIM1 protease to a chaperone associated polypeptide chain. In agreement with this hypothesis, in *E.coli* DnaK and protease La were detected in complexes which contain a non-secreted mutant form of alkaline phosphatase (Sherman and Goldberg, 1992).

Chaperone function of mt-hsp70 in the mitochondrial matrix is important for both folding of newly imported polypeptide chains (Kang *et al.*, 1990) and for the degradation of misfolded proteins. How is the fate of a chaperone associated polypeptide chain determined? Prior to degradation of the folding incompetent moiety of the tested chimeric protein, folding of the DHFR domain occurred which was shown to be mediated by molecular chaperones (Ostermann *et al.*, 1989; Kang *et al.*, 1990). Thus, misfolded segments of polypeptide chains appear to be specifically recognized by mt-hsp70 and remain bound to the chaperone protein for a prolonged time period. Distinction between a folding competent and an irreversibly misfolded polypeptide chain may occur at the level of the molecular chaperone proteins by kinetic partitioning. Polypeptide chains which fail to attain the native conformation escape aggregation by remaining for a prolonged time period in association with chaperone proteins and thereby undergo efficient degradation by the PIM1 protease. Alternatively, or in addition, sequence specific degradation signals may be recognized by the protease. Thus, a complex functional interplay must exist between the folding machinery and the proteolytic system within mitochondria and other cellular compartments.

Materials and methods

Construction of Su9(1–69)–lactalbumin

Recombinant DNA techniques were carried out as previously described (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). To generate a Su9(1–69)–lactalbumin hybrid protein, a *Hpa*I–*Pst*I fragment, which lacks DNA sequences encoding the signal sequence and three amino acids of mature α-lactalbumin, was isolated from a cDNA clone of bovine α-lactalbumin (Vilotte *et al.*, 1987). The fragment was cloned into the *Hinc*II and *Pst*I sites of pGEM4 (Promega). Then, a DNA fragment encoding the mitochondrial presequence and three amino acids of mature F_O-ATPase subunit 9 of *Neurospora crassa* was isolated by restriction digest of Su9(1–69)-cytochrome oxidase subunit II (Koll, 1991) with *Eco*RI and *Nco*I. The *Nco*I site was filled in with Klenow. The DNA fragment was cloned into the *Eco*RI and *Xba*I site, which was filled in with Klenow, of pGEM4 in front of the mature α-lactalbumin. The resulting hybrid protein consisted of the 69 amino terminal amino acids of the F_O-ATPase subunit 9 precursor fused to α-lactalbumin with a three amino acid spacer in between.

Yeast strains and growth conditions

The cultivation of yeast cells was performed according to published procedures (Rose *et al.*, 1993). The following strains from *S.cerevisiae* were used in this study: (i) 27 T1-d (ρ^- *MATa pim1::URA3 ura 3–52 trp1-Δ63 leu2-Δ1 his3-Δ200*) and the isogenic wild-type strain FY73 (ρ^+ *MATα ura3–52 his3-Δ200 Gal2*$^+$) (Van Dyck *et al.*, 1994); (ii) AB972 (*MATα trp1* ρ^0) (Rose *et al.*, 1993); (iii) PK81 [*MATα ade2–101 lys2 ura3–52 leu2–3,112 Δtrp1 ssc1-2(LEU2)*], PK83 [*MATα ade2–101 lys2 ura3–52 leu2–3,112 Δtrp1 ssc1-3(LEU2)*] and the isogenic wild-type strain PK82 (*MATα his4–713 lys2 ura3–52 Δtrp1 leu2–3,112*) (Gambill *et al.*, 1993); (iv) YNR5 (*MATa mdj1::URA3 trp1 his4::HIS3 leu2 ade2–1 can1–100* ρ^0), YNR5c (YNR5 transformed with pMDJ315) and the isogenic wild-type strain YNR7 (*MATα ura3–52 leu2 his3 trp1 lys2 suc2*) (Rowley *et al.*, 1994). If mutant strains of *S. cerevisiae* were analysed, the respective isogenic wild-type strain was used as a control. Otherwise, wild-type mitochondria were isolated from the strain D273-10B.

Import of precursor proteins into mitochondria

Mitochondria were isolated as previously described (Daum *et al.*, 1982). The genes encoding $b_2(167)^{RIC}$-DHFR and Su9(1–69)–lactalbumin were transcribed using SP6 polymerase and translated in reticulocyte lysate (Promega) as described earlier (Söllner *et al.*, 1991). Reticulocyte lysate (5–10 % of total volume) was added to import reactions (50 μg mitochondria) containing import buffer (50 mM HEPES–KOH pH 7.2, 0.5 M sorbitol, 80 mM KCl, 10 mM MgOAc, 2 mM K-phosphate, 2 mM $MnCl_2$, 3% fatty acid free BSA) in the presence of 5 mM NADH, 2.5 mM ATP and an ATP-regenerating system (10 mM phosphocreatine, 100 μg/ml creatine kinase) in a final volume of 100 μl. Import was performed for 20 min at 15°C [for $b_2(167)^{RIC}$-DHFR] or 10 min at 25°C [for Su9(1–69)–lactalbumin] and halted by the addition of 0.5 μM valinomycin and chilling on ice. Non-imported precursor proteins were digested by protease. Either trypsin (30 μg/ml) for 20 min at 0°C or proteinase K (100 μg/ml) for 30 min at 0°C were used as indicated in the figure legends. Digestion was stopped by addition of a 20-fold excess of soybean trypsin inhibitor or 2 mM PMSF. To allow degradation of the newly imported proteins, samples were incubated at 30°C or 37°C as indicated in the figure legends. At various time points mitochondria were reisolated by centrifugation for 10 min at 10 000 *g* and washed with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH pH 7.2) containing either soybean trypsin inhibitor or 1 mM PMSF. Samples were analysed by SDS–PAGE followed by laser densitometry or were used in coimmunoprecipitation experiments.

Coimmunoprecipitation of precursor proteins

Mitochondria were resuspended in IPP buffer [0.1 % (w/v) Triton X-100, 10 mM MOPS–KOH pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF] in the presence of apyrase (10 U/ml) at a concentration of 0.5 mg/ml and lysed by incubation for 10 min on ice. After a clarifiying spin for 15 min at 25 000 *g* the supernatant was incubated under gentle shaking for 60 min at 4°C with antiserum or preimmune serum coupled to protein A–sepharose. Routinely, 30 μl hsp70 specific antiserum coupled to 3 mg protein A–sepharose were added to a supernatant corresponding to 50 μg mitochondrial protein. Protein A–sepharose beads were recovered by centrifugation in an Eppendorf centrifuge, washed twice with IPP buffer and once with 10 mM MOPS–KOH pH 7.2. Finally, immunocomplexes were dissociated in SDS–PAGE sample buffer by vigorously shaking for 10 min at 4°C, incubated for 5 min at 95°C, and analysed by SDS–PAGE.

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