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Mitochondrial Hsp78, a member of the Clp/Hsp100 family in *Saccharomyces cerevisiae*, cooperates with Hsp70 in protein refolding

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Abstract The molecular chaperone protein Hsp78, a member of the Clp/Hsp100 family localized in the mitochondria of *Saccharomyces cerevisiae*, is required for maintenance of mitochondrial functions under heat stress. To characterize the biochemical mechanisms of Hsp78 function, Hsp78 was purified to homogeneity and its role in the reactivation of chemically and heat-denatured substrate protein was analyzed in vitro. Hsp78 alone was not able to mediate reactivation of firefly luciferase. Rather, efficient refolding was dependent on the simultaneous presence of Hsp78 and the mitochondrial Hsp70 machinery, composed of Ssc1p/Mdj1p/Mge1p. Bacterial DnaK/DnaJ/GrpE, which cooperates with the Hsp78 homolog, ClpB in *Escherichia coli*, could not substitute for the mitochondrial Hsp70 system. However, efficient Hsp78-dependent refolding of luciferase was observed if DnaK was replaced by Ssc1p in these experiments, suggesting a specific functional interaction of both chaperone proteins. These findings establish the cooperation of Hsp78 with the Hsp70 machinery in the refolding of heat-inactivated proteins and demonstrate a conserved mode of action of ClpB homologs.

Key words: Chaperone; Hsp100 family; Protein refolding; Mitochondrion; *Saccharomyces cerevisiae*

1. Introduction

All organisms respond to a variety of stress conditions with the transient acceleration of the synthesis of a group of proteins, collectively referred to as stress or heat shock proteins. Most heat shock proteins act either as molecular chaperones, which stabilize protein substrates and promote their folding, or as proteases, which mediate degradation of irreversibly denatured substrate proteins. Hsp100/Clp proteins comprise an evolutionary conserved family of heat shock proteins possessing ATP-dependent chaperone activity (for reviews see [1,2]). They are thought to mediate protein unfolding as well as the disassembly of protein aggregates and oligomers. While some Hsp100/Clp family members act only as molecular chaperone proteins and confer cellular thermotolerance, others can function both as chaperone proteins and subunits of ATP-dependent proteases.

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Abbreviations: BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride

Hsp100/Clp proteins of the B-subtype appear to be involved in stress thermotolerance. Several members of this subfamily have been identified in various organisms, including Hsp104 and Hsp78 in *Saccharomyces cerevisiae* [3,4], ClpB in various bacteria [5–7]. First evidence for a role of these proteins in conferring cellular thermotolerance was obtained for Hsp104 in *S. cerevisiae*. The survival rate of cells exposed to extreme temperatures was found to be severely reduced in *Δhsp104* strain when compared to wild type [3,8]. Electron-dense structures, presumably representing aggregated proteins, accumulated under these conditions in both wild type and *hsp104* mutant cells. These aggregates were eliminated in wild type but not in *Δhsp104* cells [9]. Similar to Hsp104, the *Escherichia coli* homolog ClpB also maintains cellular thermotolerance [5,6]. Thermally aggregated proteins were dissolved in wild type cells but not in a *clpB* mutant strain upon shifting the cells to lower temperature [10]. While these results suggest a role of Hsp104 and ClpB in the reactivation of heat-denatured proteins, their exact mode of action at the molecular level remains unknown.

The thermotolerance of *Δhsp104* cells can be restored by overproduction of Hsp70 proteins pointing to a functional relationship between Hsp104 and Hsp70 proteins [11]. The reactivation of heat-denatured proteins by purified Hsp104, Hsp70 and Hsp40 proteins in a reconstituted in vitro system established the functional cooperation between both these chaperone systems [12]. Similarly, both ClpB and the DnaK system were found to be required for the solubilization of protein aggregates accumulating upon heat shock in *E. coli* [13] and for the reactivation of heat-denatured proteins in a reconstituted bacterial system [14–17].

In *S. cerevisiae*, a second member of the B-type subfamily of Hsp100/Clp proteins, termed Hsp78, was localized in the mitochondrial matrix space [4]. Hsp78 shares 50% sequence identity to *E. coli* ClpB, 44% sequence identity to yeast cytosolic Hsp104, and 43% sequence identity to *E. coli* ClpA [4]. When expressed in the cytosol, Hsp78 can substitute for a loss of its cytosolic homolog Hsp104, suggesting a conserved mode of action [18]. While disruption of the *HSP78* gene did not affect cell growth at temperatures up to 37°C [4], Hsp78 was found to be crucial for the maintenance of respiratory competence and mitochondrial genome integrity at extreme temperatures [18]. Mitochondrial protein synthesis, which was inactivated under stress conditions, is efficiently restored upon recovery after heat stress in wild type but not in cells lacking Hsp78 [18]. Thus, similar to Hsp104, Hsp78 appears to ensure the reactivation of heat-inactivated mitochondrial proteins, rather than protecting them from heat inactivation

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[18]. However, the molecular mechanism of Hsp78 action remains unclear.

In this report, we have purified Hsp78 to homogeneity and analyzed its chaperone activity in vitro.

2. Materials and methods

2.1. Purification of Hsp78

A derivative of the *S. cerevisiae* wild type strain W303-1B carrying pYES2-ctHSP78 [18] was grown in YP-galactose medium at 30°C to an optical density 4.5 in a 10 l fermentor (150 g of cells). Spheroplasts were generated by incubation of the cells with zymolyase (Seikagaku Corp.) as described [20]. Spheroplasts were isolated by centrifugation and resuspended in 50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) containing in addition the following protease inhibitors: pepstatin 12 μg/ml, leupeptin 7 μg/ml, benzamidine 1 mM. Spheroplasts were disrupted in a French Press (Aminco) at 20 000 psi. After a clarifying spin, proteins were precipitated with ammonium sulfate (0.28 g/ml), resuspended in 50 mM Tris–HCl pH 7.4, 50 mM KCl, 5 mM β-mercaptoethanol, 10% (v/v) glycerol, 1 mM PMSF, dialyzed and loaded onto a Q-Sepharose column. Proteins were eluted with a linear salt gradient (50–400 mM KCl). Fractions containing Hsp78 were combined and dialyzed to 20 mM phosphate buffer pH 6.8, 10% (v/v) glycerol, 5 mM β-mercaptoethanol and loaded onto a P11 phosphocellulose column (Whatman). Proteins were eluted with a linear salt gradient (0–250 mM KCl). Fractions containing Hsp78 were applied to a hydroxylapatite column (Bio-Rad) equilibrated with the same buffer. Hsp78 was eluted with a linear potassium phosphate gradient (20–150 mM) and dialyzed against 50 mM Tris–HCl pH 7.4, 100 mM KCl, 5 mM β-mercaptoethanol, 10% (v/v) glycerol, frozen in liquid nitrogen and stored at −70°C.

2.2. Purification of other proteins

Published protocols were used for the purification of *E. coli* DnaK, DnaJ, GrpE [21], *E. coli* ClpB [22], and yeast Ssc1p [23]. Purified Mdj1p and Mge1p were kindly provided by Dr. Olivier Deloche (University of Geneve) and Frank King (University of Gdansk). Purified Ssa1p and Sis1p were a kind gift of Dr. Elisabeth Craig (University of Wisconsin, Madison, WI, USA). Firefly luciferase (E 1701) was purchased from Promega.

Protein concentrations were determined with the Bio-Rad assay system, using bovine serum albumin (BSA) as a standard. Molar concentrations are given on the basis of a hexameric structure of Hsp104, Hsp78 and ClpB and of a monomeric structure of other proteins.

2.3. Refolding of urea-denatured luciferase

Firefly luciferase (4 μM) was denatured for 3 h at 30°C in 40 mM Tris–HCl, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, 15 mM Mg-acetate containing 6 M urea. For refolding, luciferase (50 nM) was incubated at 25°C in the same buffer (40 μl) lacking urea supplemented with ATP (5 mM), an ATP regenerating system (10 mM phosphocreatine, 100 μg/ml phosphocreatine kinase), 0.15 mg/ml BSA and chaperone proteins as indicated in the figure legends. The luciferase activity was determined in a Beckman scintillation counter using the Luciferase Assay System (Promega E1500).

2.4. Refolding of heat-denatured luciferase

The reactivation of heat-denatured luciferase was analyzed as described [24].

3. Results

3.1. Purification of Hsp78 from S. cerevisiae

Nuclear-encoded Hsp78 is synthesized in the cytosol and targeted by a N-terminal presequence to mitochondria [19]. To facilitate purification of Hsp78, the mature form of Hsp78 lacking the mitochondrial leader sequence was overproduced from a yeast multicopy plasmid allowing protein expression under the control of a galactose-inducible promoter (pYES2-ctHSP78) [18]. Hsp78 protein was subsequently purified to homogeneity as described in Section 2 (Fig. 1, lane 3). Isolation of Hsp78 was monitored by Western blot analysis with antibodies directed against the C-terminus of Hsp78. Sequencing of the purified protein identified the amino acid residues MRMDPN at the N-terminus. In agreement with the cloning procedure [18], the purified polypeptide initiated at the fourth amino acid residue of mature Hsp78.

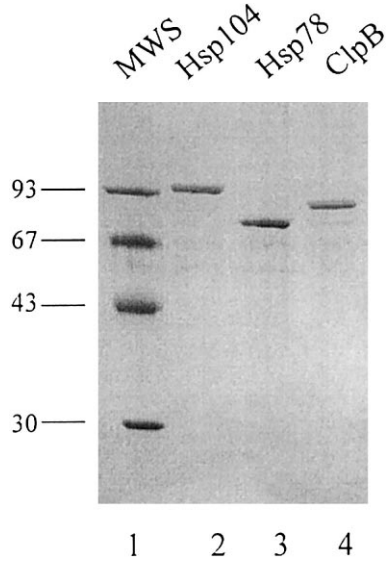

Fig. 1. Purified Hsp100/Clp proteins. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane 1, protein molecular weight standards (kDa); lane 2, Hsp104 (1 μg); lane 3, Hsp78 (1 μg); lane 4, ClpB (1 μg).

3.2. Cooperation of Hsp78 with the mitochondrial Hsp70 machinery, Ssc1p, Mdj1p and Mge1p in luciferase refolding

Binding of Hsp78 to misfolded polypeptides in the mitochondrial matrix space and its stabilization against aggregation provided first evidence for a chaperone activity [19]. We therefore tested the ability of Hsp78 to mediate the refolding of urea-denatured firefly luciferase in vitro. Hsp78 was found to interact with denatured but not with native luciferase using an enzyme-linked immunosorbent binding assay (data not shown). However, it did not promote the reactivation of luciferase upon further incubation in the presence of ATP (Fig. 2A). Similarly, the mitochondrial Hsp70 machinery composed of Ssc1p and its two co-chaperones Mdj1p and Mge1p was also ineffective in luciferase refolding under these conditions (Fig. 2A). In contrast, we observed efficient reactivation of luciferase in the presence of both Hsp78 and the Hsp70 system (Fig. 2A). Homologous chaperone proteins from *E. coli*, namely ClpB, DnaK, DnaJ, GrpE, were found to be similarly active in luciferase refolding in parallel experiments (Fig. 2B). These results are reminiscent of previous studies using homologous chaperone proteins from yeast cytosol (Hsp104 and Ssa1p/Ydj1p) and from bacteria (ClpB and DnaK/DnaJ/GrpE), indicating a conserved mode of action [12,14–16].

3.3. Specificity of the functional cooperation of Hsp100 and Hsp70 proteins

To further characterize the functional interplay of these

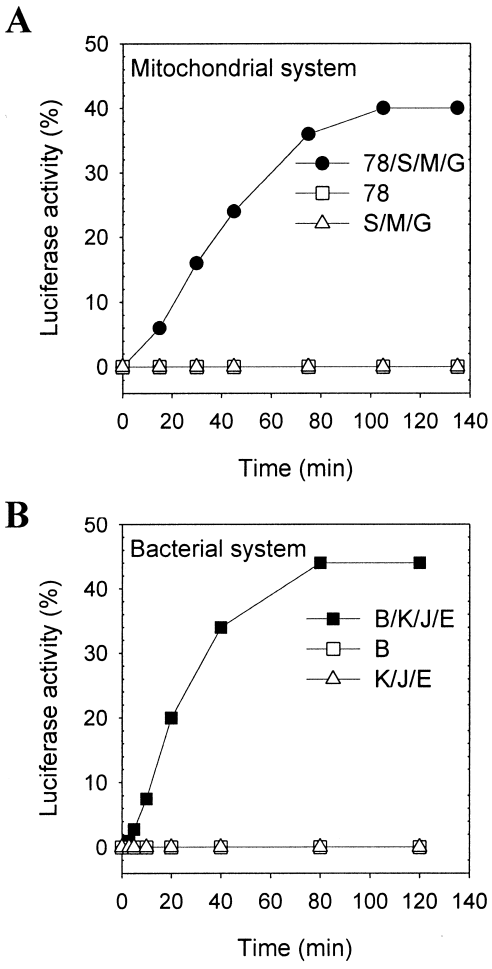

Fig. 2. Chaperone-mediated refolding of firefly luciferase. A: Mitochondrial chaperone system; B: bacterial chaperone system. Refolding of urea-denatured luciferase was examined in the presence of different combinations of Hsp78 (0.33 μM; '78'), Ssc1p (1 μM; 'S'), Mdj1p (0.2 μM; 'M') and Mge1p (0.1 μM; 'G') or ClpB (0.33 μM; 'B'), DnaK (1 μM; 'K'), DnaJ (0.2 μM; 'J') and GrpE (1 μM; 'E') as indicated. The activity of native luciferase was set to 100%.

chaperone proteins, luciferase refolding was analyzed in the presence of Hsp100 and Hsp70 proteins isolated from different cellular compartments and organisms. Neither mitochondrial Hsp78, cytosolic Hsp104 nor bacterial ClpB alone was able to reactivate luciferase in vitro (Fig. 3). In each case, refolding was dependent on the presence of a Hsp70 system. The cooperation of both chaperone systems appears to be specific: Hsp78 promoted the reactivation of luciferase only in the presence of the mitochondrial Hsp70 system, which could not be replaced by the bacterial homologs, DnaK, DnaJ and GrpE, or the yeast cytosolic Hsp70 proteins, Ssa1p and Sis1p (Fig. 3A). Similarly, Hsp104-mediated luciferase was dependent on Ssa1p and Sis1p, and its activity was impaired in the presence of the mitochondrial or bacterial Hsp70 systems (Fig. 3B). On the other hand, ClpB cooperated efficiently with mitochondrial Ssc1p, Mdj1p, Mge1p and bac-

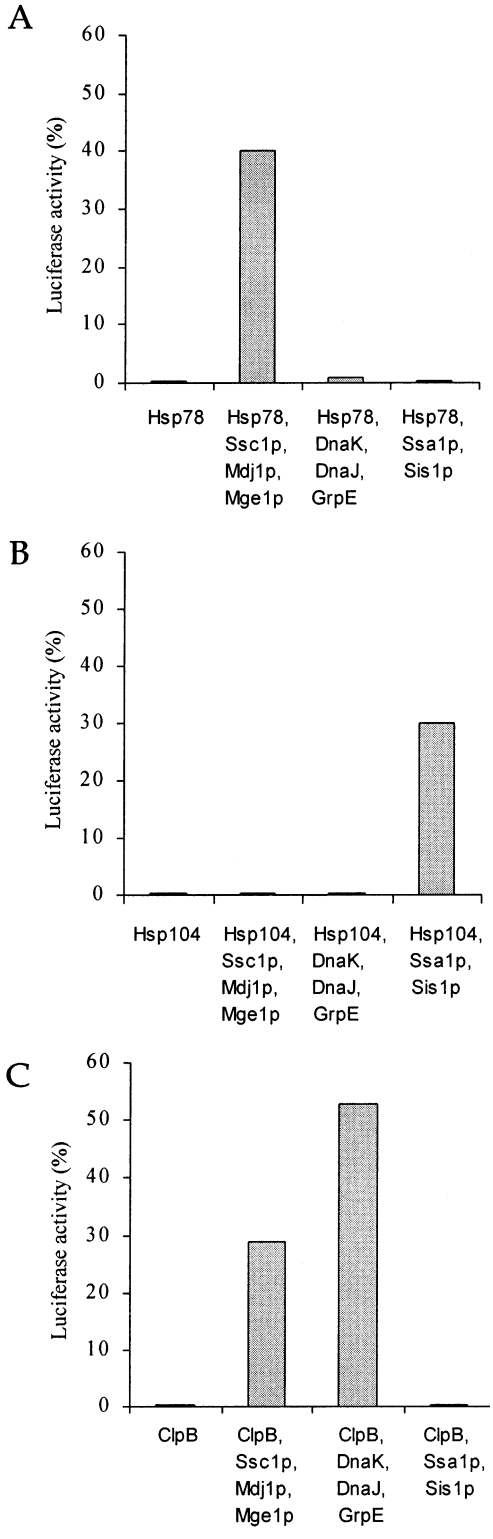

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Fig. 3. Cooperation of Hsp100 and Hsp70 chaperone systems isolated from various cellular compartments and organisms. The cooperation of Hsp100 and Hsp70 proteins in the refolding of chemically denatured luciferase was analyzed using (A) mitochondrial Hsp78, (B) cytosolic Hsp104, (C) bacterial ClpB and Hsp70 systems from yeast mitochondria, *E. coli* and the yeast cytosol. Refolding of chemically denatured luciferase (50 nM) was monitored in the presence of various combinations of chaperone proteins as indicated: Hsp78 (0.33 μM), ClpB (0.33 μM), Hsp104 (0.33 μM), Ssc1p (1 μM), DnaK (1 μM), Ssa1p (1 μM), Sis1p (0.2 μM), Mdj1p (0.2 μM), DnaJ (0.2 μM), Mge1p (0.1 μM) and GrpE (1 μM). Following 2 h incubation luciferase activity was determined. The activity of untreated native luciferase was set to 100%.

terial DnaK, DnaJ, GrpE, but not with yeast cytosolic Ssa1p and Sis1p (Fig. 3C).

3.4. Functional interactions between Ssc1p and Hsp78 are required for luciferase refolding

To determine which component of the mitochondrial Hsp70 system is critical for interactions with Hsp78, the refolding of luciferase was performed in the presence or absence of individual components from either mitochondrial or bacterial Hsp70 chaperone system. Individual replacement of either Mdj1p or Mge1p by their bacterial homologs DnaJ and GrpE, respectively, did not significantly impair luciferase refolding (Fig. 4, lanes 3 and 4). We also observed efficient reactivation if both Mdj1p and Mge1p were simultaneously substituted by their bacterial homologs (Fig. 4, lane 7). However, refolding was completely dependent on the presence of mitochondrial Ssc1p (Fig. 4, lanes 2, 5, 6 and 8), and bacterial DnaK could not substitute for Ssc1p in these experiments. We conclude that the functional cooperation of mitochondrial chaperone proteins Ssc1p and Hsp78 is crucial for the refolding of luciferase.

3.5. Hsp78-mediated reactivation of heat-denatured proteins

Hsp78 is required for thermoprotection of mitochondrial functions [18]. We therefore examined the ability of Hsp78 to protect thermolabile firefly luciferase against heat inactivation. Luciferase was heat-treated at 43°C for 10 min in the absence of molecular chaperones, or in the presence of either Hsp78 or Ssc1p, Mdj1p and Mge1p and luciferase activity was determined immediately after incubation. While luciferase was completely inactivated at 43°C in the absence of chaperone proteins, the stability of luciferase was only slightly increased upon the addition of Hsp78 (Fig. 5, lanes 2, 3; black bars) or Ssc1p, Mdj1p and Mge1p (Fig. 5, lanes 4, 5; black bars). Also presence of all four chaperone proteins did not significantly increase thermal stability of luciferase (Fig. 5, lane 6; black bar).

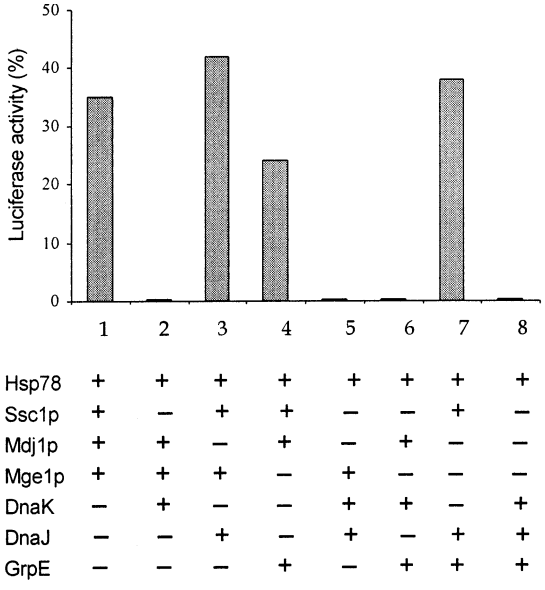

Fig. 4. Ssc1p is crucial for the cooperation of Hsp78 with the mitochondrial Hsp70 machinery. Reactivation of luciferase was examined as described in Fig. 3. Hsp78 and combinations of mitochondrial and bacterial chaperones were present as indicated.

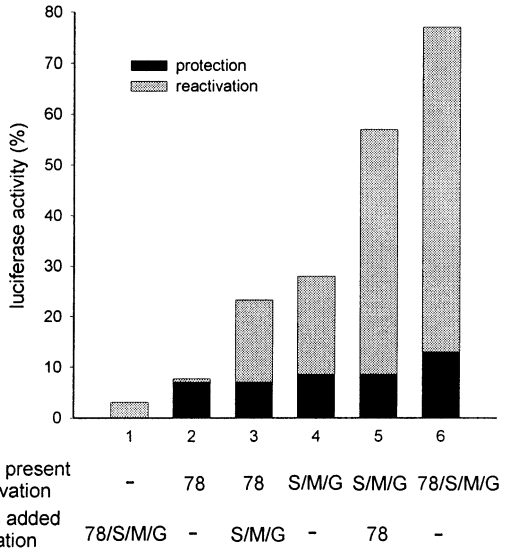

Fig. 5. Hsp78 cooperates with mitochondrial chaperones in the reactivation of heat-denatured luciferase. Luciferase was heat-treated at 43°C for 10 min in the presence or absence of the indicated chaperones (0.33 μM Hsp78; or 1 μM Ssc1p, 0.2 μM Mdj1p and 0.1 μM Mge1p). Aliquots were withdrawn and luciferase activity was determined (protection; black bars). The reactions were then transferred to 25°C and supplemented with the indicated chaperones (0.33 μM Hsp78; or 1 μM Ssc1p, 0.2 μM Mdj1p and 0.1 μM Mge1p). Following a further incubation at 25°C for 2 h, luciferase activity was determined (reactivation; gray bars). The activity of untreated native luciferase was set to 100%.

Even though Hsp78 and Hsp70 proteins were incapable of preventing heat denaturation of luciferase in vitro, the cooperate action of both chaperone systems was found to mediate efficient reactivation of the heat-denatured enzyme. For reactivation the luciferase samples were transferred to 25°C. The highest efficiency of luciferase reactivation was observed if both chaperone systems were present during the inactivation step (Fig. 5, lane 6; gray bars). In contrast, the addition of Hsp78 along with Ssc1p, Mdj1p and Mge1p after heat treatment did not allow refolding of luciferase (Fig. 5, lane 1). Presence of either Hsp78 or the Hsp70 system during heat inactivation step significantly increased the later reactivation in the presence of both chaperone systems (Fig. 5, lanes 3 and 5; gray bars). However, it should be noted that the Hsp70 system mediated reactivation of luciferase to some extent even when Hsp78 was omitted from the reaction (Fig. 5, lane 4). These results demonstrate the cooperative action of both Hsp78 and the Hsp70 system in the reactivation of heat-denatured proteins.

4. Discussion

Mitochondrial chaperone proteins are essential for mitochondrial biogenesis as well as the protection and maintenance of mitochondrial proteins during stress conditions. In this report, we isolated a new mitochondrial chaperone Hsp78 and investigated its activity in vitro. Hsp78 was shown to cooperate in refolding of chemically and thermally denatured luciferase with chaperones from mitochondrial Hsp70 system. Our in vitro studies provide a rational for previous in vivo experiments, which assigned a crucial function for Hsp78 in the thermoprotection of mitochondria. The maintenance of

respiratory competence and mitochondrial genome integrity was found to be dependent on Hsp78 under severe temperature stress [18]. Hsp78 did not prevent thermal inactivation of the mitochondrial protein synthesis, but rather allowed reactivation of this process upon recovery from the heat stress [18]. Moreover, it was shown that Hsp78 does not act alone, and other heat shock proteins are involved in the reactivation of mitochondrial protein synthesis [18]. Our findings are in agreement with these in vivo results. First, Hsp78 did not protect luciferase against heat denaturation but allowed its reactivation. Second, our results identified the mitochondrial Hsp70 system as an important component for mediating the refolding of heat-denatured proteins. This conclusion is also consistent with the observation that Mdj1p, the co-chaperone of Ssc1p, is required to prevent heat-induced protein aggregation in mitochondria [25,26]. This suggests that Hsp78 and the mitochondrial Hsp70 system together form a chaperone network in the mitochondrial matrix, which allows the reactivation of heat-denatured proteins and confers thermoprotection to mitochondria.

Experiments with the replacement of various components of the mitochondrial chaperone network by homologous proteins from an other cellular compartment (yeast cytosol) or organism (*E. coli*) pointed to a specific functional interaction of Hsp78 and the mitochondrial Hsp70 protein Ssc1p during the coordinated action of both chaperone systems. Hsp78 was only capable of reactivating denatured luciferase in cooperation with the bacterial Hsp70 system, if DnaK was replaced by Ssc1p. The mitochondrial co-chaperones Mdj1p and Mge1p have previously been demonstrated to functionally cooperate with bacterial DnaK in vivo [27] and to stimulate the ATPase activity of DnaK in vitro [28,29]. The lack of Hsp78-dependent refolding of luciferase in the presence of the bacterial Hsp70 system is therefore not due to the inability of the heterologs systems composed of DnaK/Mdj1p/GrpE or DnaK/DnaJ/Mge1p to function as a chaperone machine. Rather, it appears to reflect the loss of a specific functional cooperation between mitochondrial Ssc1p and Hsp78.

In summary, data presented in this paper establish the cooperation of Hsp78 with the Hsp70 machinery (Ssc1p/Mdj1p/Mge1p) in the refolding of denatured substrate protein, which presumably mimics the reactions performed by these chaperones in yeast mitochondria in vivo.

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