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

Joanna Krzewska\textsuperscript{a}, Thomas Langer\textsuperscript{b}, Krzysztof Liberek\textsuperscript{a,*}

\textsuperscript{a}Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdansk, Gdansk, Poland
\textsuperscript{b}Institut für Physiologische Chemie der Universität München, München, Germany

Received 14 November 2000; revised 19 December 2000; accepted 20 December 2000

First published online 4 January 2001

Edited by Horst Feldmann

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 thermostability, others can function both as chaperone proteins and subunits of ATP-dependent proteases.

*Corresponding author. Fax: (48)-58-301 92 22.
E-mail: liberek@biotech.univ.gda.pl

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

0014-5793/01/$20.00 © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

PH: S0014-5793(00)02423-6
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-Cl, 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 (Amino2) at 20000 psi. After a clarifying spin, proteins were precipitated with ammonium sulfate (0.28 g/ml), resuspended in 50 mM Tris-Cl, 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 Mono Q column (Whatman). Proteins were eluted with a linear salt gradient (20–150 mM) and dialyzed against 50 mM Tris-Cl, pH 8.4, 100 mM KCl, 5 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 (Amino2) at 20000 psi. After a clarifying spin, proteins were precipitated with ammonium sulfate (0.28 g/ml), resuspended in 50 mM Tris-Cl, 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 Mono Q column (Whatman). Proteins were eluted with a linear salt gradient (0–250 mM KCl). Fractions containing Hsp78 were applied to a hydroxyapatite column (Bio-Rad) equilibrated with the same buffer. Hsp78 was eluted with a linear potassium phosphate gradient (50–400 mM KCl) and dialyzed against 50 mM Tris-Cl, 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 Geneva) 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-Cl, 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 reaction 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 over-produced from a yeast multicopy plasmid allowing protein expression under the control of a galactoside-inducible pro-

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-

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%.

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).

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 re-activation 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 vitro [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.

Acknowledgements: We thank Drs. Elisabeth Craig, Olivier Deloche and Frank King for their gifts of purified proteins and plasmids. We are also grateful to Drs. Jaroslaw Marszalek, Igor Konieczny and Frank King for discussions and critical reading of the manuscript. This work was supported by the Grant 6P04B02317 from the Polish State Committee for Scientific Research.

References