Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulators

Ellen J. Levy[a], John McCarty[b], Bernd Bukau[b], William J. Chirico[a,*]

[a]*Department of Anatomy and Cell Biology, Box 5, State University of New York Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA*
[b]*Zentrum für Molekuläre Biologie, Universität Heidelberg, INF 282, 69120 Heidelberg, Germany*

Received 9 November 1994; revised version received 12 May 1995

Abstract We have reconstituted an ATP-dependent protein folding machinery using purified yeast cytosolic proteins. The *S. cerevisiae* Hsp70 Ssa1p and the DnaJ homolog Ydj1p refolded denatured firefly luciferase. In *E. coli*, efficient refolding of luciferase requires the Hsp70 DnaK and two modulators, DnaJ and GrpE, that synergistically stimulate its ATPase activity. Exchanging DnaJ homologs between the *S. cerevisiae* and *E. coli* systems revealed that their ability to stimulate Hsp70 ATPase activity was conserved. In contrast, GrpE further stimulated only DnaK's ATPase activity. Efficient refolding of luciferase by Ssa1p and DnaJ, but not by DnaK and Ydj1p, suggests that a compatible Hsp70/DnaJ homolog pair can act as a protein folding machinery.

Key words: Protein folding; Heat-shock protein

1. Introduction

Hsp70 chaperones participate in many cellular protein folding processes (reviewed in [1]). They bind short, extended, hydrophobic peptides that resemble the hydrophobic cores of proteins [2–4]. Hsp70 chaperones may participate in protein folding by binding to exposed hydrophobic segments of proteins, thereby preventing the aggregation of unfolded proteins [5,6] or nascent chains [7]. Proteins may fold either through repeated interactions with Hsp70 chaperone machineries, GroEL/ES-like chaperonin systems [8,9], or both.

The Hsp70 chaperone of *E. coli*, DnaK, and its two modulators, DnaJ and GrpE, are stress proteins that were originally discovered because mutations in their genes led to defects in bacteriophage l DNA replication (reviewed in [10]). DnaJ and GrpE together increase the low intrinsic ATPase activity of DnaK as much as 50-fold; alone, GrpE increases activity by less than 2-fold [11], whereas DnaJ increases activity by as much as 13-fold (J. McCarty, A. Buchberger and B. Bukau, manuscript in prep.). DnaJ promotes the conversion of bound ATP to ADP, whereas GrpE promotes dissociation of either ATP or ADP from DnaK [11]. Substrate release from and conformational changes of DnaK are coupled to ATP binding or hydrolysis [12–14]. In yeast, at least eight Hsp70 chaperones have been identified and members of families are located in the cytosol, mitochondria, and endoplasmic reticulum (reviewed in [15]). Two cytosolic Hsp70 chaperones, Ssa1p and Ssa2p, were shown to facilitate the translocation of protein precursors into endoplasmic reticulum [16,17] and mitochondria [17,18]. These proteins are 98% homologous to each other and 55% homologous to DnaK [15]. DnaK [19,20], but not other chaperones tested [20,21], can partially substitute for Ssa1p in in vitro translocation assays.

In addition to acting as a modulator of DnaK ATPase activity, the 41 kDa DnaJ protein itself acts as a molecular chaperone [5] and targets DnaK to substrates [6]. Several eukaryotic *dnaJ* homologs, including *S. cerevisiae MAS5/YDJ1* [22,23] and *MDJ1* [24], have been identified (reviewed in [25,26]). DnaJ homologs show greater overall sequence variation than Hsp70 chaperones, but they contain a characteristic, highly conserved N-terminal 'J' domain that may permit interactions with Hsp70 chaperones [27]. DnaJ is 32% homologous to Ydj1p and can partially substitute for Ydj1p in the import of some proteins into endoplasmic reticulum and mitochondria in vivo [28]. Ydj1p greatly stimulates the ATPase activity of and protein substrate dissociation from Ssa1p [29].

Amino acid identity among bacterial GrpE homologs ranges from 9 to 30%, lower than among either DnaK or DnaJ homologs [30]. GrpE forms stable complexes with DnaK that are disrupted by addition of ATP [31]. GrpE binds to DnaK at a loop consisting of amino acids 28–33 near its ATP binding site [32]. Although this loop is conserved among Hsp70s, amino acid identity within the loop is higher between prokaryotic and mitochondrial Hsp70s than between cytosolic/endoplasmic reticulum and prokaryotic/mitochondrial Hsp70s. The amino acid sequence of a recently identified *S. cerevisiae* GrpE homolog is 34% identical to *E. coli* GrpE and contains mitochondrial targeting information [33–35]. Cytosolic GrpE homologs have not been reported.

Firefly luciferase has been used as a model substrate for studying chaperone requirements for protein folding [6,36–39]. The concerted action of DnaK, DnaJ, and GrpE results in the refolding of heat-denatured luciferase in vivo and in vitro [6]. GroEL/ES is required for efficient refolding of denatured luciferase in vivo [6], but not in vitro [6,36]. Denatured luciferase can also be refolded using rabbit reticulocyte lysates [37,38] and either purified Hsp70 and Hsp90 [38] or TRiC, a chaperonin of the eukaryotic cytosol that is distantly related to GroEL [36].

We report here the reconstitution of an in vitro protein folding system using proteins purified from yeast cytosol. We also examined the functional conservation of *E. coli* and *S. cerevisiae* cytosolic Hsp70 chaperones and their modulators by studying the hydrolysis of ATP and refolding of luciferase. We found that DnaJ or Ydj1p stimulated the ATPase activity of either DnaK or Ssa1p. GrpE and either DnaJ or Ydj1p acted syner-

*Corresponding author. Fax: (1) (718) 270 3732.

Abbreviation: Hsp, Heat-shock protein.

SSDI 0014-5793(95)00704-0

gistically to stimulate DnaK ATPase activity. However, GrpE, together with either DnaJ or Ydj1p, did not act synergistically to stimulate Ssa1p ATPase activity. Although Ydj1p stimulated DnaK ATPase activity, it poorly substituted for DnaJ during DnaK-dependent refolding of luciferase. Ssa1p and either Ydj1p or DnaJ refolded luciferase, suggesting that a compatible Hsp70/DnaJ-homolog pair serves as a protein folding machinery.

2. Materials and methods

2.1. Proteins

DnaK [6,31], DnaJ [40] and Ydj1p [29] were purified as previously described. Ssa1p was purified [16] from MW141 cells [41]. Ssa1p or DnaK was then applied to a MonoQ HR 5/5 column (Pharmacia) equilibrated with 20 mM Hepes (pH 7.5), 100 mM potassium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol (DTT). Ssa1p or DnaK was eluted using a linear gradient of potassium acetate from 0.1 to 1.0 M. Hsp70 preparations obtained using MonoQ columns after ATP affinity chromatography have lower ATPase activity than those purified without using a final MonoQ column [42]. Purity was assessed using SDS-PAGE [16]. GrpE was overexpressed in *E. coli* as the N-terminal hexahistidine fusion protein and metal chelate-affinity purified [43]. Storage buffer for DnaK, Ssa1p, and Ydj1p was buffer K (25 mM Hepes (pH 7.4), 50 mM KCl, 2 mM DTT and 10% (w/v) glycerol); for DnaJ was 40 mM KH_2PO_4 (pH 6.8), 100 mM KCl, 5 mM DTT, 0.1 mM EDTA, 0.05% Brij 58, and 10% glycerol; and for GrpE was 25 mM Hepes (pH 7.4), 300 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5 mM EDTA, and 10% glycerol. Protein concentrations were determined using the method of Bradford [44] (Bio-Rad Protein Assay) with bovine gamma globulin as a standard.

2.2. ATPase assays

Proteins were assayed for ATPase activity in 10 μl reactions containing 5 μl of ATPase reaction buffer and 5 μl of either purified proteins or compensating amounts of storage buffers. ATPase reaction buffer contained 60 mM Hepes (pH 7.4), 50 mM KCl, 8 mM DTT, 22 mM $MgCl_2$, 0.2 mM ATP, and 1 μCi of [α-^{32}P]ATP (30 Ci/mmol, Amersham). DnaK (Ssa1p or Ydj1p), DnaJ, and GrpE contributed 3.09 μl, 0.66 μl, and 1.25 μl, respectively, towards assay volume. GrpE was diluted 8.66-fold into Buffer K before use in ATPase assays. Reactions were incubated at 30°C. At various times, 1 μl was removed from reactions and spotted on thin layer chromatographic plates (Baker-flex Cellulose PEI). Plates were developed using 1 M formic acid/0.5 M LiCl [45] and radiolabeled nucleotides visualized using autoradiography. Spots corresponding to ATP and ADP were removed and radioactivity measured using a scintillation counter. Percent ATP hydrolyzed was calculated as [1 − (cpm ATP/cpm (ATP + ADP)) × 100] and those values at time zero were subtracted from later time points. All data points are the average of at least two replicates.

2.3. Binding assays

Reactions (12 μl) containing various combinations of DnaK (6.9 μM), Ssa1p (6.9 μM), and GrpE (63.6 μM) were incubated for 30 min at 25°C. After the incubation, 3 μl sample buffer (67% glycerol and 0.08% bromophenol blue) were added to each reaction and the resulting mixture was separated immediately on a 12% native polyacrylamide gel with a stacking gel at 4°C [32]. Proteins were visualized using Coomassie Blue.

2.4. Luciferase refolding assay

Firefly luciferase (Sigma) was stored at 4 mg/ml in 1 M glycylglycine (pH 7.4) and assayed as previously described [32]. Storage buffer for DnaK, Ydj1p, and Ssa1p (above) was modified by lowering the concentration of glycerol to 1% (Buffer K-1%). GrpE was diluted 8.66-fold into Buffer K-1% before use in luciferase refolding assays. Luciferase was denatured by diluting 2-fold into 1 M glycylglycine and then 6.4-fold into unfolding buffer (25 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 6 M guanidine hydrochloride). The resulting mixture was incubated at 25°C for 40 min. Denatured luciferase (1 μl) was then diluted (time zero) into a 125 μl refolding reaction containing refolding buffer (25 mM Hepes (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT), purified chaperones or compensating amounts of storage buffers, and 1 mM ATP. Refolding reactions were incubated at 30°C. At the indicated times, 1 μl of each reaction was diluted into 60 μl of luciferase assay mixture (50 μl of Luciferase Assay Substrate Solution (Promega) and 10 μl of 1 × Lysis Reagent (Promega) containing 6 mg/ml bovine serum albumin). Activity was measured using an Optocomp I luminometer (MGM Instruments Inc., Hamden, CT). Native luciferase activity was measured after first diluting the stock solution 12.8-fold into 1 M glycylglycine and then diluting 125-fold into refolding buffer. All activities were calculated as a percent of the native luciferase activity. All data points are the average of at least two replicates.

3. Results

3.1. Ydj1p stimulates the ATPase activity of DnaK

To gain insight into the level of evolutionary conservation among Hsp70 chaperones and modulators, we studied the hydrolysis of ATP and refolding of luciferase. We first measured the rates of DnaK-catalyzed hydrolysis of ATP in the presence or absence of DnaJ and GrpE (Fig. 1A). DnaK catalyzed the hydrolysis of ATP at a low rate of 0.27 nmol/min/mg of protein (Fig. 1A, curve K). GrpE and DnaJ increased the rate 1.7- and 3.6-fold, respectively (Fig. 1A, K + E and K + J). Together DnaJ and GrpE increased the rate of ATP hydrolysis of DnaK 36-fold (Fig. 1A, curve K + J + E). In the presence of DnaJ and GrpE alone, the amount of ATP hydrolyzed was negligible (data not shown). These results confirm the cooperative ability of GrpE and DnaJ to stimulate the ATPase activity of DnaK reported by Liberek et al. [11]. However, the results reported here differ in two respects. The intrinsic ATPase activity of our MonoQ-purified DnaK (see Section 2) was 13-fold lower and DnaJ stimulatory activity was at least 2-fold higher [11]. The significantly greater stimulation of DnaK ATPase activity by DnaJ than by GrpE alone agrees with recent results from other laboratories (J. McCarty, A. Buchberger and B. Bukau, manuscript in prep.; R. McMacken, pers. commun.).

After establishing rates of ATP hydrolysis in the presence of prokaryotic Hsp70 chaperones and modulators, we tested whether the eukaryotic DnaJ homolog Ydj1p could enhance the ATPase activity of DnaK (Fig. 1B). The rate of ATP hydrolysis of DnaK was increased 3.5-fold by Ydj1p (Fig. 1B, curves K and K + Y). Furthermore, GrpE increased this rate to 13-fold higher than DnaK alone (Fig. 1B, curve K + Y + E). The rate of ATP hydrolysis in the presence of Ydj1p and GrpE alone was negligible (data not shown). The results demonstrate that the eukaryotic DnaJ homolog, Ydj1p, can substitute for DnaJ in stimulating ATPase activity of DnaK. This suggests that the DnaK-DnaJ interaction domains responsible for stimulating ATP hydrolysis are evolutionarily conserved. The ability of Ydj1p and GrpE to act together synergistically to stimulate DnaK ATPase activity suggests that the binding of GrpE to DnaK is not precluded by DnaK's association with Ydj1p. Furthermore, Ydj1p does not obviate the requirement of DnaK for GrpE to obtain maximal stimulation of ATPase activity.

3.2. DnaJ, but not GrpE, greatly stimulates the ATPase activity of Ssa1p

The abilities of Ydj1p, DnaJ, and GrpE to modulate the ATPase activity of Ssa1p were evaluated (Fig. 1C,D). The rate of ATP hydrolysis in the presence of Ssa1p and GrpE was indistinguishable from that of Ssa1p alone (Fig. 1C, curves S and S + E; 0.3 nmol/min/mg). Ydj1p stimulated the ATPase

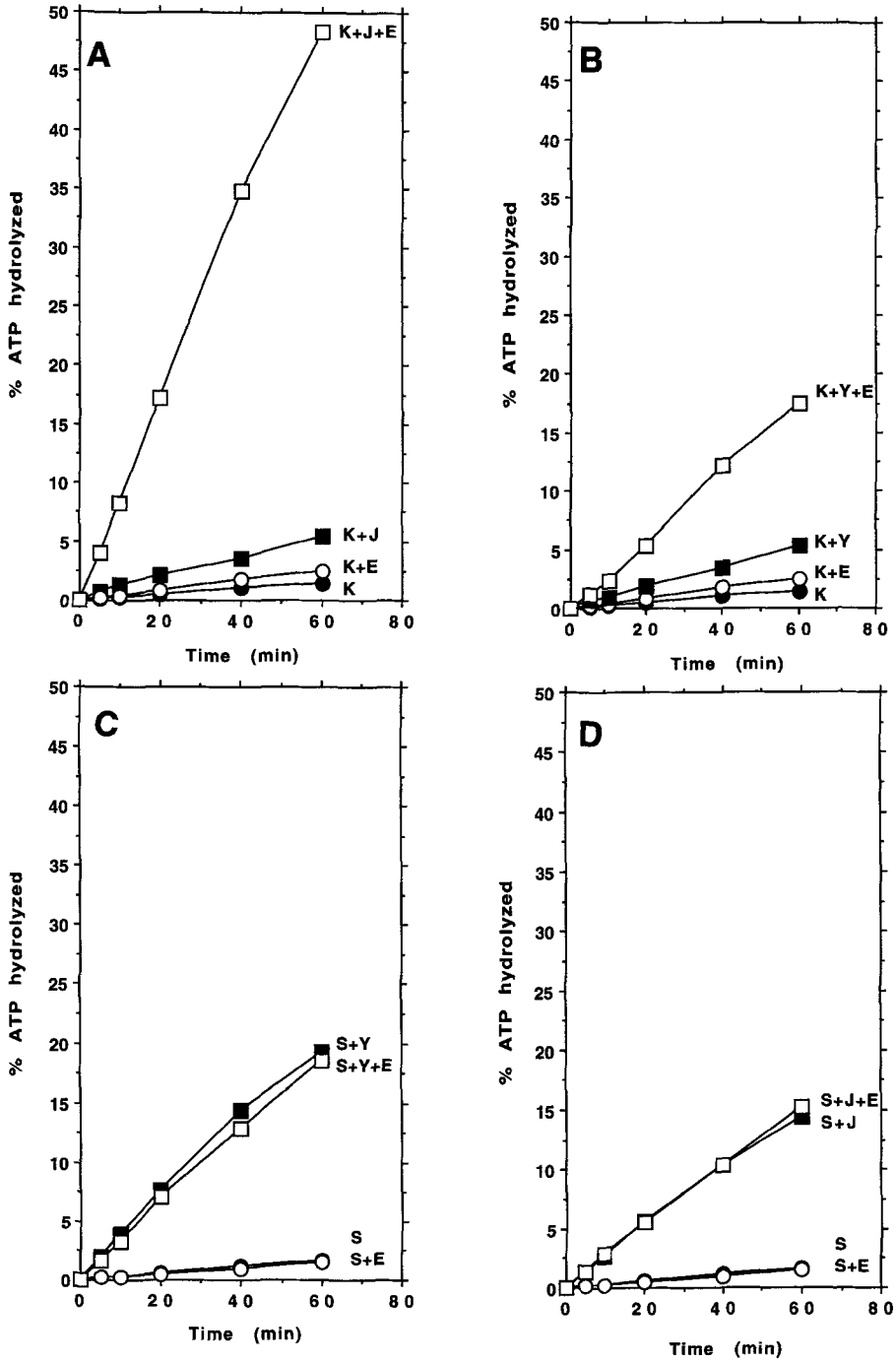

Fig. 1. Modulation of ATPase activity of DnaK or Ssa1p by DnaJ, Ydj1p and GrpE.DnaK (A and B) or ssa1p (C and D) was incubated in ATPase assays alone or with the indicated modulators. DnaK, Ssa1p, DnaJ, Ydj1p and GrpE are abbreviated as K, S, J, Y and E, respectively. The concentration of each protein was 1.28 μM.

activity of Ssa1p 13-fold, but GrpE did not further stimulate the activity (Fig. 1C, curves S + Y and S + Y + E). Our results are consistent with the 10-fold stimulation of Ssa1p ATPase activity by Ydj1p previously reported [29].

In the presence of DnaJ, the ATPase activity of Ssa1p was 9-fold higher than in its absence (Fig. 1D, curves S and S + J). GrpE did not act synergistically with DnaJ to stimulate the ATPase activity of Ssa1p (Fig. 1D, curve S + J + E).

We tested the possibility that higher concentrations of GrpE might be necessary to cooperatively enhance the ATPase activity of Ssa1p in the presence of either Ydj1p or DnaJ. A 4-fold increase in the concentration of GrpE did not increase the rate of ATP hydrolysis of Ssa1p in the presence of either Ydj1p or DnaJ (data not shown). Taken together, the inability of GrpE to act synergistically with either DnaJ or Ydj1p to stimulate Ssa1p ATPase activity suggests that interactions between

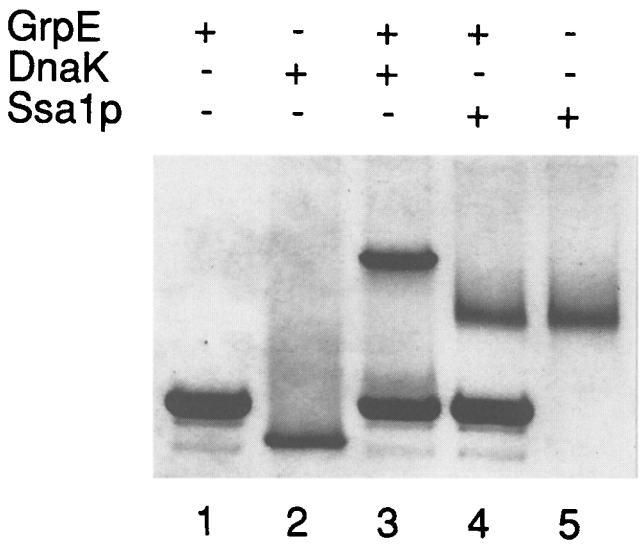

Fig. 2. Gel shift assay of GrpE binding to DnaK or Ssa1p. GrpE (63.6 μM) was incubated for 30 min at 25°C in a 12 μl reaction alone (lane 1), with DnaK (6.9 μM, lane 3), or with Ssa1p (6.9 μM, lane 4). DnaK (lane 2) or Ssa1p (lane 5) at the same concentrations were also incubated alone. The resulting mixtures were separated on a native polyacrylamide gel and proteins visualized using Coomassie Blue.

Hsp70 chaperones and GrpE are less evolutionarily conserved than those involving DnaJ homologs.

3.3. GrpE binds DnaK, but not Ssa1p

We used native polyacrylamide gel electrophoresis to test the possibility that the failure of GrpE to substantially increase the ATPase activity of Ssa1p might result from the lack of interaction between these proteins (Fig. 2). Buchberger et al. [32] previously demonstrated that GrpE-DnaK complexes can be detected using this technique. Purified GrpE (lane 1), DnaK (lane 2), and Ssa1p (lane 5) migrated as single bands. When DnaK and GrpE were incubated together, a new, more slowly migrating band appeared and the intensities of DnaK and GrpE bands correspondingly decreased, indicating that they formed a stable complex as previously demonstrated (lane 3 and [32]). In contrast, no indication of GrpE-Ssa1p complexes was found (lane 4). The lack of a stable interaction between GrpE and Ssa1p may partly explain the absence of stimulation of Ssa1p ATPase activity by GrpE.

3.4. Ydj1p or DnaJ and Ssa1p refold luciferase, but Ydj1p poorly substitutes for DnaJ during DnaK/DnaJ/GrpE-dependent refolding of luciferase

We extended the study of the conservation of modulator function by confirming the refolding of denatured firefly luciferase using DnaK, DnaJ, and GrpE and attempting to reconstitute an Ssa1p-dependent protein refolding system. Together DnaK, DnaJ, and GrpE refolded denaturant-unfolded luciferase to 70% of its native activity as previously reported (Fig. 3A, curve K1 + J + E, and [32]). Without GrpE, DnaK and DnaJ at these concentrations did not efficiently refold denatured luciferase (Fig. 3A, curve K1 + J). However, doubling the concentration of DnaK improved GrpE-independent refolding 4-fold (Fig. 3A, curve K2 + J). Although Ydj1p acted together with GrpE to synergistically stimulate the ATPase activity of DnaK, it poorly substituted for DnaJ during luciferase refolding in the presence of DnaK and GrpE even at a 10-fold higher concentration than required for DnaJ (Fig. 3B, curve K1 + Y + E). Ydj1p and DnaK also did not promote substantial refolding of luciferase (Fig. 3B, curve K1 + Y).

The concerted action of DnaK, DnaJ and GrpE yields optimal refolding, but the absence of any reported cytosolic GrpE homologs led us to test whether Ssa1p and Ydj1p are sufficient to refold denatured luciferase. Ssa1p and Ydj1p refolded denaturant-unfolded luciferase to about 30% of its native activity (Fig. 3B, curve S2 + Y). A 2-fold decrease in the concentration of Ssa1p still allowed substantial refolding (Fig. 3B, curve S1 + Y). DnaJ substituted for Ydj1p during Ssa1p-dependent refolding and yielded a level of refolding approaching that of the optimal DnaK, DnaJ, and GrpE system (Fig. 3A, curve S1 + J). Ssa1p alone did not refold luciferase. Addition of GrpE to either the Ssa1p-Ydj1p or Ssa1p-DnaJ systems did not further increase luciferase refolding (data not shown), consistent with the lack of a stable interaction between Ssa1p and GrpE. The minimum requirement for protein refolding using luciferase as a model substrate appears to be a compatible Hsp70-DnaJ homolog couple.

4. Discussion

We reconstituted an Hsp70 protein refolding system using purified *S. cerevisiae* cytosolic proteins and compared its ATPase and luciferase refolding activities with the DnaK-based chaperone machinery previously reconstituted from *E. coli*. We found that Ssa1p and either Ydj1p or DnaJ, which are 32% identical, refold luciferase. DnaK and DnaJ together refold luciferase; however, optimal refolding requires GrpE. The DnaK-DnaJ chaperone pair has also been shown to monomerize RepA dimers [46] and to reactivate heat-denatured RNA polymerase [47]. Members of the DnaJ protein family were shown previously to bind denatured proteins, prevent their aggregation, and target them to Hsp70s. Ssa1p-dependent luciferase refolding is more efficient with DnaJ than with Ydj1p, suggesting that DnaJ may be superior to Ydj1p in any of these respects. Ydj1p substituted poorly for DnaJ in luciferase refolding in the presence of DnaK and GrpE. The ability of DnaJ and Ssa1p to refold luciferase efficiently in vitro suggests that the *dnaJ* gene product may act as a chaperone and modulator of cytosolic Hsp70s without a GrpE counterpart during the in vivo functional complementation of the *ydj1* null [28].

Requirements for modulators in ATP hydrolysis, however, appear to be less strict than in luciferase refolding. DnaJ or Ydj1p stimulated the ATPase activity of either DnaK or Ssa1p. However, Ssa1p was stimulated more by either DnaJ or Ydj1p than was DnaK. Perhaps Ssa1p exchanges nucleotides more efficiently than does DnaK, thereby lessening the requirement for a GrpE-like factor.

The inability of GrpE to act synergistically with a DnaJ homolog to stimulate Ssa1p ATPase activity may stem from the lack of stable interactions between GrpE and Ssa1p. We showed that DnaK, but not Ssa1p, forms stable complexes with GrpE. Of the seven amino acids comprising the GrpE binding loop of DnaK, four amino acids of a similar loop of Ssa1p are significantly different. A compatible GrpE-like factor in yeast cytosol may be required for optimal chaperone activity. However, until such a factor is identified in yeast cytosol, the possi-

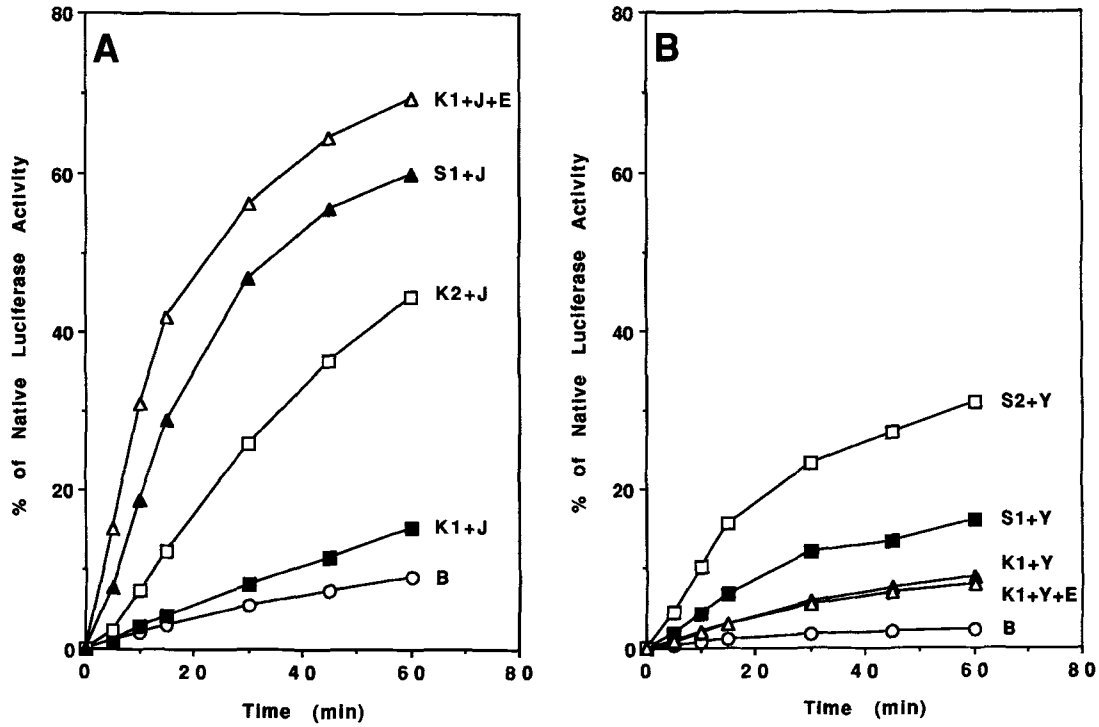

Fig. 3. Refolding of denatured luciferase by DnaK or Ssa1p and DnaJ, Ydj1p, or GrpE.Denatured luciferase (40 nM) was incubated in refolding buffer (see Section 2) with either no protein (B) or the indicated combinations of DnaK (K1, 0.8 μM; K2, 1.6 μM), DnaJ (0.16 μM), GrpE (0.8 μM), Ssa1p (S1, 0.8 μM; S2, 1.6 μM) and Ydj1p (1.6 μM).(A) The buffer system contained 0.29% glycerol and 0.0008% Brij 58. (B) The buffer system contained 0.17% glycerol. All chaperone-dependent refolding required ATP. Abbreviations are the same as in Fig. 1 legend.

bility remains that Ssa1p and Ydj1p function without a GrpE-like factor in this compartment.

The requirements for chaperone-dependent protein folding have been studied in vivo and in vitro using a variety of model proteins including firefly luciferase [6,36–39]. We demonstrated above that the combination of two eukaryotic chaperones, Ssa1p and Ydj1p, is sufficient to refold denaturant-unfolded luciferase. Ydj1p used in these studies was overexpressed in and purified from *E. coli* and therefore did not contain the carboxyl terminal farnesyl group added in yeast. Optimal protein folding and other chaperone-dependent activities in yeast cytosol may involve farnesylated Ydj1p, different pairs of Hsp70s and DnaJ homologs [48], a putative GrpE-like factor, or other chaperones, such as Hsp90 or TRiC. Hsp70, a DnaJ homolog, and TRiC, but not Hsp90, were reported recently to be involved in the co-translational folding of luciferase in reticulocyte lysates [39]. Frydman et al. [36] demonstrated refolding of denaturant-unfolded luciferase using only purified TRiC from bovine testis. In contrast, Schumacher et al. [38] showed that luciferase refolding activity in reticulocyte lysates is independent of TRiC, and that purified Hsp70 and Hsp90 together are sufficient to refold heat-denatured luciferase. However, the Hsp70/Hsp90:luciferase molar ratio [38] was 800-fold higher than the Ssa1p/Ydj1p:luciferase molar ratio used here. The human DnaJ homolog, HDJ-1, did not improve the refolding activity of Hsp70 and Hsp90 [38]. Two differences exist between our work and that of Schumacher et al. [38] that might explain the difference in chaperone requirements. We denatured luciferase using guanidine hydrochloride, whereas they used a mild heat treatment. As previously suggested [38], heat-denatured luciferase may be only partially unfolded and therefore have different chaperone requirements for refolding. Furthermore, we maintain nonionic detergent and glycerol concentrations below levels that promote substantial chaperone-independent refolding of denaturant-unfolded luciferase (Schröder and Bukau, unpublished). Concentrations of nonionic detergent (0.1%) and glycerol (1%) used by Schumacher et al. [38] during refolding may have obscured a DnaJ homolog requirement. The exact requirements for chaperones and modulators may depend on the particular protein substrate being studied and the degree to which it is unfolded.

Acknowledgements: We thank Dr. Elizabeth Craig for strain MW141 and Josh Rothman for purifying DnaK. We also thank Drs. John Lewis, Robert Garofalo, and Herbert Weissbach for reviewing the manuscript. This work was supported by a Junior Faculty Research Award from the American Cancer Society (#JFRA-341) and a grant from the National Science Foundation (MCB-9118464) to W.J. Chirico.

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