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Hsp104, Hsp70, and Hsp40: A Novel Chaperone System that Rescues Previously Aggregated Proteins

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Summary

Hsp104 is a stress tolerance factor that promotes the reactivation of heat-damaged proteins in yeast by an unknown mechanism. Herein, we demonstrate that Hsp104 functions in this process directly. Unlike other chaperones, Hsp104 does not prevent the aggregation of denatured proteins. However, in concert with Hsp40 and Hsp70, Hsp104 can reactivate proteins that have been denatured and allowed to aggregate, substrates refractory to the action of other chaperones. Hsp104 cooperates with the chaperones present in reticulocyte lysates but not with DnaK of *E. coli*. We conclude that Hsp104 has a protein remodeling activity that acts on trapped, aggregated proteins and requires specific interactions with conventional chaperones to promote refolding of the intermediates it produces.

Introduction

Hsp104 is the most crucial thermotolerance-related protein of *Saccharomyces cerevisiae*, enhancing survival after exposure to extreme heat or high concentrations of ethanol by >1000-fold (Sanchez and Lindquist, 1990; Sanchez et al., 1992). Ultrastructural analysis of cells exposed to a sublethal heat shock reveals electron-dense abnormalities, presumably aggregated proteins (Parsell et al., 1994b) indistinguishable in wild-type and *hsp104* mutants. The difference between these cells becomes apparent only during recovery: abnormalities persist in the absence of Hsp104 but are rapidly eliminated in wild-type cells. Similarly, in cells expressing bacterial luciferase, reporter enzyme activity and solubility are lost at the same rate and to the same extent with or without Hsp104 (Parsell et al., 1994b). However, during recovery at normal temperatures, luciferase activity and solubility are restored only in cells expressing Hsp104.

There is, however, no evidence that Hsp104 directly disaggregates denatured proteins, and there are many reasons to doubt it. Other Hsps prevent aggregation by stabilizing nonnative proteins in the soluble state or promoting their degradation. The in vitro reactivation of aggregated substrates by chaperones has been observed in only a few special cases with very specific substrates (Hwang et al., 1990; Skowyra et al., 1990; Ziemienowicz et al., 1993), and never with aggregates formed from completely denatured proteins. Indeed, the prevention of aggregation is generally the key element in chaperone-assisted reactivation of unfolded proteins in vitro. Thus, Hsp104's ability to promote the resolubilization of heat-damaged proteins in vivo might reflect a capacity to alter directly or indirectly the nature of aggregates during their formation, rather than a capacity to act post facto on proteins that have already aggregated.

Hsp104 is a member of the HSP100/Clp family of oligomeric ATPases (Schirmer et al., 1996). *Escherichia coli* representatives of this family, notably ClpA and ClpX, are involved in protein turnover. Neither ClpA nor ClpX has intrinsic proteolytic activity. Rather, both proteins confer ATP-dependent turnover of their substrates through a physical association with an unrelated oligomeric protease ClpP (Katayama-Fujimura et al., 1987; Hwang et al., 1988; Wojtkowiak et al., 1993). In the absence of this protease partner protein, ClpA disassembles inactive RepA dimers, releasing active monomers (Wickner et al., 1994; Pak and Wickner, 1997), and ClpX disassembles the MuA tetramer/DNA complex, releasing free functional MuA monomers (Levchenko et al., 1995). Hsp104 has no detectable role in protein degradation (Parsell et al., 1993), but structural conservation among the HSP100s suggests that all family members may employ a common molecular mechanism with distinct consequences. Thus, Hsp104 may also act by pulling apart oligomeric assemblages, not of specific folded proteins, but of a broad spectrum of heat-damaged, inactivated proteins. Whether Hsp104 has such a function can only be determined by reconstitution of Hsp104 function in vitro using purified components.

We now demonstrate that Hsp104 has the ability to rescue chemically denatured proteins from previously formed high–molecular weight aggregates. Reactivation of such substrates by conventional chaperones has never been reported. Refolding from the aggregated state requires not only Hsp104, but additional specific chaperones—*SSA*-encoded Hsp70, a DnaK homolog, and *YDJ1*-encoded Hsp40, a DnaJ homolog. Together, these proteins comprise a protein refolding machine with novel activities.

Results

Hsp104's Influence on Aggregation and Refolding

First we tested the ability of Hsp104 to suppress aggregation and to promote refolding of chemically denatured proteins—two hallmark activities of conventional molecular chaperones. As reported by others, aggregation of denatured firefly luciferase was suppressed (Figure 1A) by *E. coli* GroEL (Buchberger et al., 1996) as well as by yeast Ssa1 (Hsp70) and Ydj1 (Hsp40) in the presence of ATP (Cyr, 1995). However, Hsp104 at a 5-fold molar excess failed to suppress the aggregation of denatured luciferase. When aggregation was analyzed by an independent method—differential centrifugation—even at Hsp104:luciferase ratios of 20:1, only a very small quantity of luciferase remained soluble, and only in the presence of ATP and ATPγS (Figure 1B).

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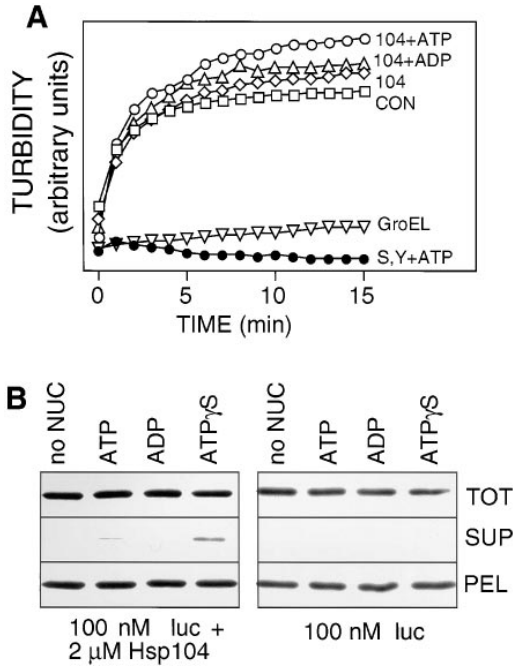

Figure 1. Hsp104 Does Not Block Aggregation of Denatured Luciferase

(A) Unfolded luciferase was diluted to 0.6 μM into buffer alone (open squares), buffer containing 3 μM Hsp104 with no nucleotide (diamonds), 5 mM ATP (open circles), or 5 mM ADP (triangles). Dilution into 0.6 μM *E. coli* GroEL 14-mers (inverted triangles) and 3 μM each of the yeast chaperones Ssa1 and Ydj1 with 5 mM ATP (filled circles) were used as positive controls for the suppression of aggregation.

(B) Unfolded luciferase was diluted to 100 nM in the presence of the indicated nucleotide (5 mM), with or without 2 μM Hsp104. After 15 min incubation at 25°C the reactions were centrifuged at 14,000 × g for 10 min. Equal portions of the total reaction (TOT), supernatant (SUP), and pellet (PEL) were analyzed by SDS-PAGE and Western blot with anti-firefly luciferase antibody.

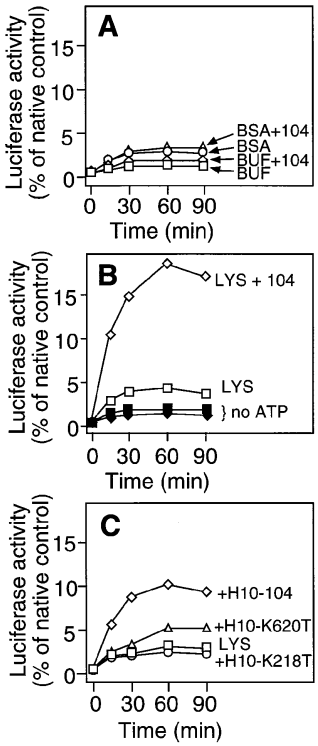

Figure 2. Hsp104-Mediated Refolding Requires Protein Cofactors and ATP

(A) Unfolded luciferase was diluted to 20 nM into buffers containing 5 mM ATP and an ATP-regenerating system without Hsp104 (open squares), with 5 μg Hsp104 (diamonds), with 100 μg BSA (open circles), or with 100 μg BSA and 5 μg Hsp104 (triangles). Refolding was measured as enzyme activity detected in aliquots withdrawn from reactions at the indicated times.

(B) Unfolded luciferase was diluted as in (A) into buffer containing 100 μg of yeast lysate lacking endogenous Hsp104 (open squares) and into the same amount of lysate supplemented with 5 μg Hsp104 (diamonds). ATP-requirement for refolding was demonstrated by omitting ATP from identical reactions (filled symbols).

(C) To test the refolding function of mutant derivatives of Hsp104, 20 nM denatured luciferase was refolded in Hsp104-deficient lysate alone (open squares) or lysate supplemented with 5 μg His-tagged wild-type Hsp104 (+H10–104; diamonds), or with mutant derivatives of Hsp104 carrying a lysine-to-threonine substitution in the first nucleotide-binding domain (+H10-K218T; open circles), or a lysine-to-threonine substitution in the second nucleotide-binding domain (H10-K620; triangles). All reactions contained 5 mM ATP and an ATP-regenerating system.

Purified Hsp104 by itself promoted only slight reactivation of denatured luciferase (Figure 2A). However, when Hsp104 was added to a cell-free lysate prepared from an *HSP104*-deletion strain, it strongly stimulated the reactivation of denatured luciferase in an ATP-dependent manner (Figure 2B). Demonstrating that the ATP requirement was related specifically to Hsp104 function, proteins carrying single amino acid substitutions in either of Hsp104's two nucleotide-binding domains (NBDs)—K218T (NBD1) and K620T (NBD2)—did not support efficient refolding (Figure 2C). Thus, *HSP104* mutations that impair thermotolerance (Parsell et al., 1991) and reduce ATP hydrolysis (Schirmer et al., 1998) also impair Hsp104-mediated refolding.

Hsp70 and Hsp40 Homologs Are Required for Hsp104-Mediated Refolding

To identify components involved in Hsp104-stimulated refolding, lysates were fractionated by a variety of chromatographic techniques. No individual fractions could support refolding with Hsp104, suggesting that at least two factors are required and that they fractionate independently (data not shown). To identify candidate factors, proteins that physically interact with Hsp104 were captured by affinity chromatography with immobilized His-tagged Hsp104. Ydj1, a yeast homolog of human Hsp40 and *E. coli* DnaJ (Caplan and Douglas, 1991), coeluted with Hsp104 (Figure 3A) irrespective of the inclusion of nucleotide or nucleotide analogs (not shown). No other chaperone among those tested by Western blotting showed a specific interaction with Hsp104.

The physical interaction between Hsp104 and Ydj1 was not robust; <1% of the total Ydj1 was recovered, with an excess of His-tagged Hsp104. However, chaperone complexes may be intrinsically dynamic in nature and difficult to isolate. The functional significance of Ydj1 in Hsp104-mediated protein refolding was tested in cell-free lysates prepared from yeast strains lacking

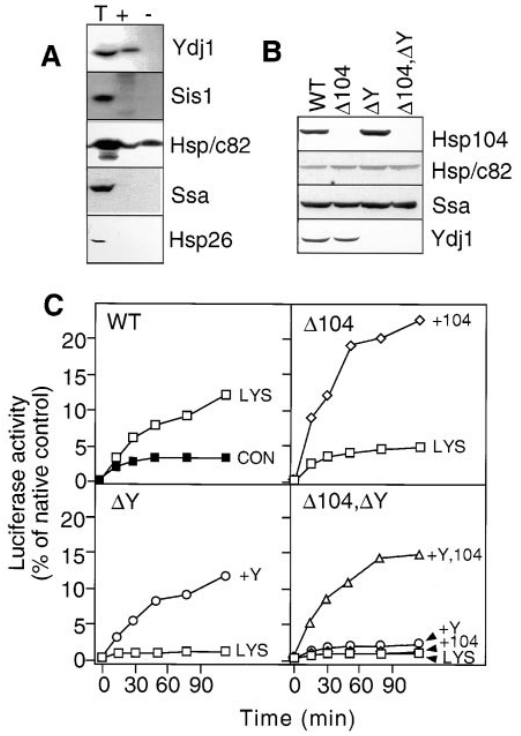

Figure 3. Ydj1 Physically Interacts with Hsp104 and Is Necessary for Hsp104-Dependent Protein Refolding

(A) His-tagged Hsp104 was immobilized on Ni^{2+} resin and incubated with yeast lysate as described in Experimental Procedures. Aliquots of lysate representing 5% of total protein (T) and 25% of eluates from columns with (+) or without (-) bound Hsp104 were resolved by SDS-PAGE. Blotted proteins were probed with antibodies specific for the indicated proteins.

(B) Lysates were prepared from YPH499 (WT) and isogenic strains deficient in the expression of Hsp104 (Δ104), Ydj1 (ΔY), or both proteins (Δ104,ΔY). Fifty micrograms of each lysate was analyzed by SDS-PAGE and Western blotting using antisera specific for the indicated proteins.

(C) Unfolded luciferase (20 nM) was refolded in buffer containing 100 μg BSA (filled squares) or 100 μg unsupplemented lysates (open squares), lysates supplemented with 2.5 μg Hsp104 (diamonds), 5 μg Ydj1 (open circles), or both Hsp104 and Ydj1 (triangles). All reactions contained 5 mM ATP and an ATP regenerating system.

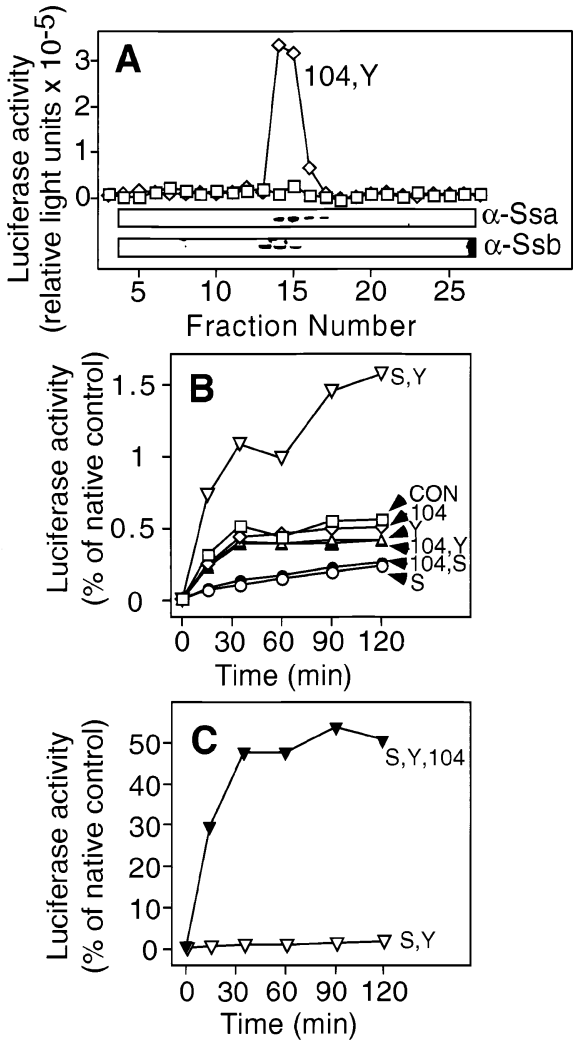

Figure 4. *SSA*-Encoded Hsp70 Is Also Required for Hsp104-Dependent Refolding

(A) Lysate was fractionated by anion exchange chromatography, as described in Experimental Procedures. An equal volume of each fraction was analyzed by SDS-PAGE and Western blotting with antisera specific for *SSB*- and *SSA*-encoded Hsp70. Each fraction was desalted into refolding buffer and tested for refolding of denatured luciferase (20 nM) alone (open squares) or supplemented with 2.25 μg Hsp104 and 1.75 μg Ydj1 (diamonds). All reactions contained 5 mM ATP and an ATP regenerating system.

(B) Unfolded luciferase (20 nM) was refolded in 5 mM ATP without chaperones (CON; open squares), or with 1 μM each of Ssa1 alone (S; open circles), Ssa1 and Hsp104 (S,104; filled circles), Ydj1 alone (Y; open triangles), Ydj1 and Hsp104 (Y,104; filled triangles), Hsp104 alone (104; diamonds), and Ssa1 and Ydj1 (S,Y; inverted triangles).

(C) Refolding by Ssa1 and Ydj1 (open inverted triangles) in (B), is reiterated on a different vertical scale for comparison with refolding in reactions containing 1 μM each of Ssa1, Ydj1, and Hsp104 together (S,Y,104; closed inverted triangles).

endogenous Hsp104, Ydj1, or both. Western blotting of lysates prepared from stationary phase wild-type and deletion strains indicated that the mutations had no substantial effects on the accumulation of other chaperones (Figure 3B). When denatured luciferase was added, the efficiency of refolding was diminished in each mutant lysate (Figure 3C). However, refolding was restored by the addition of the purified missing protein(s). Thus, although the physical interaction between Ydj1 and Hsp104 is weak in vitro, their functional cooperation in protein refolding is clear. Nevertheless, purified Hsp104 and Ydj1 together did not promote refolding of denatured luciferase (see Figure 4B).

With two components of the refolding reaction in hand, anion exchange chromatography was employed to search for other factors required for Hsp104-mediated renaturation. When each column fraction was tested for its ability to support luciferase reactivation in the presence of Hsp104 and Ydj1, a single peak of refolding activity was observed (Figure 4A). Others have shown that Ydj1 interacts specifically with *SSA*-encoded Hsp70 to prevent substrate aggregation and to promote refolding (Cyr and Douglas, 1994; Cyr, 1995; Levy et al., 1995), and that *SSA*-encoded Hsp70s are required for the refolding of guanidinium-HCl-unfolded luciferase in yeast

extracts (Bush and Meyer, 1996). Western blot analysis of the column fractions indicated that *SSA*-encoded Hsp70 was indeed present in the active column fractions, while *SSB*-encoded Hsp70, a functionally distinct Hsp70 of the yeast cytosol (Cyr and Douglas, 1994; Becker et al., 1996), eluted slightly ahead.

When luciferase refolding was tested with purified Hsp104, Ydj1, and Ssa1, the essential features of refolding in yeast lysates were reproduced (Figure 4B). None of the individual chaperones promoted refolding. Neither did the combinations of Hsp104 and Ydj1 or Hsp104 and Ssa1. Ssa1 and Ydj1 together supported a very modest level of refolding, as previously observed by others (Levy et al., 1995). The addition of Hsp104 to reactions containing both Ssa1 and Ydj1 increased the yield of refolded protein 30-fold (Figure 4C; note the change in scale from Figure 4B).

Rescue of Previously Aggregated Proteins

In vivo, Hsp104 is necessary not only for the reactivation of a reporter enzyme but also for its retrieval from an aggregated state (Parsell et al., 1994b). We therefore analyzed Hsp104's capacity to promote refolding of denatured luciferase that had already begun to aggregate. Initially, we employed heat-denatured aggregated luciferase. Hsp104 strongly enhanced the refolding of substrates formed in this manner, but the extent of aggregation was difficult to control, and yields of refolded luciferase varied considerably from experiment to experiment (data not shown). To provide a more uniform substrate, we therefore turned to chemically denatured luciferase. Even so, aggregates inexorably increased in size, became too large for standard sizing techniques, and eventually became irretrievable by any chaperones. Modification of the solvent by the addition of osmolytes diminishes aggregation, but these additives interfere with subsequent chaperone-dependent refolding (Singer and Lindquist, 1998). Instead, we exploited the observation that for some unfolded proteins, cold temperature diminishes the extent of aggregation (Xie and Wetlaufer, 1996) without the introduction of potentially problematic solutes.

Denatured luciferase diluted into ice-cold refolding buffer in the absence of chaperones produced a nonnative substrate that could be held for a prolonged period in a state amenable to refolding upon return to 25°C. Efficient refolding occurred only when Hsp104, Ssa1, and Ydj1 were added together (Figure 5). Remarkably, removal of particulate material from suspension, by passage through a 0.22 μm filter (shown) or by sedimentation (not shown), eliminated about 30% of the refoldable luciferase, indicating that even very large aggregates are substrates for refolding by the combined action of these proteins.

When addition of Hsp104, Ssa1, and Ydj1 was delayed after warming to 25°C, the substrate gradually lost the capacity to be refolded upon subsequent addition of the complete set of chaperones (Figure 5). However, if Ssa1 and Ydj1 were present, nonnative luciferase was stabilized in a state from which it could be refolded on subsequent addition of Hsp104. Hsp104, however, had no capacity to stabilize luciferase for reactivation upon subsequent addition of Ssa1 and Ydj1.

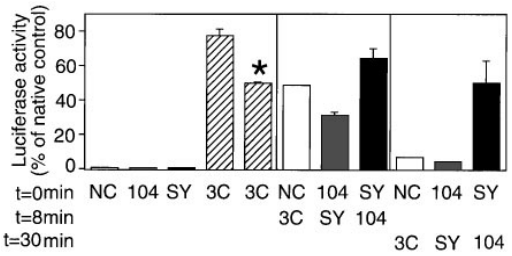

Figure 5. Refolding of Luciferase Substrate Stabilized by Cold

Unfolded luciferase was diluted to 100 nM into ice-cold refolding buffer and incubated on ice for 30 min. To determine the amount of refoldable luciferase that was associated with large particles, one aliquot of this material (indicated by an asterisk) was fractionated by passage through a precooled 0.22 μm filter prior to refolding. On ice, ATP (5 mM) was added together with 1 μM each of Hsp104, Ssa1, and Ydj1 (3C = 3 chaperones; hatched bars), with buffer only (NC = no chaperones; open bars), with 1 μM Hsp104 (104; gray bars), or 1 μM each of Ssa1 and Ydj1 (SY; solid bars). Reactions were warmed to 25°C and yield of refolded luciferase was determined after 90 min. To determine how rapidly substrate becomes irretrievable, the indicated additional chaperones (1 μM) were added to incomplete reactions at the indicated times and refolding was determined 90 min after addition.

To investigate the nature of the refolding substrate formed in the cold, the nonparticulate material was size-fractionated. A continuum of aggregated forms was recovered (Figure 6A). These fractions provided refolding substrate in various stages of aggregation, allowing us to assess directly the relationship between aggregation state and chaperone requirements for refolding. Very small aggregates, with apparent molecular sizes of ≤400 kDa, could be refolded with Ssa1 and Ydj1 alone, albeit with a very low yield (Figure 6A, inset shows refolding by Ssa1 and Ydj1 on an expanded scale). Even with such small luciferase aggregates, Hsp104 greatly enhanced refolding. Most notably, larger aggregates were completely dependent upon Hsp104 for refolding.

We next investigated substrates formed from chemically denatured luciferase at higher temperatures in the presence of Ssa1 and Ydj1, which are highly dependent on Hsp104 for refolding. When denatured luciferase was dispersed into 25°C buffer containing ATP and a 5-fold molar excess of Ssa1 and Ydj1 (sufficient to block turbidity; see Figure 1A), it was stabilized in a retrievable nonnative state for extended periods, such that upon addition of Hsp104, refolding was enhanced >500-fold, with >50% yield (not shown). After incubation at 25°C for 30 min, reactions containing Ssa1, Ydj1, and ATP together with unfolded luciferase were size-fractionated to analyze the aggregation state of the remaining soluble nonnative luciferase. Inactive luciferase was found in aggregates with a size greater than 400 kDa (Figure 6B). Refolding of this substrate was highly Hsp104-dependent but required the readdition of all three chaperones. Based on densitometry, yields from reactions containing all three chaperones range from ~10% from fractions near the void volume to >80% for fractions eluting with an apparent molecular weight of 600 kDa.

Next we asked if Hsp104 could cooperate with Ssa1 and Ydj1 to further limit aggregation of nonnative proteins. To do so, denatured luciferase was diluted into a

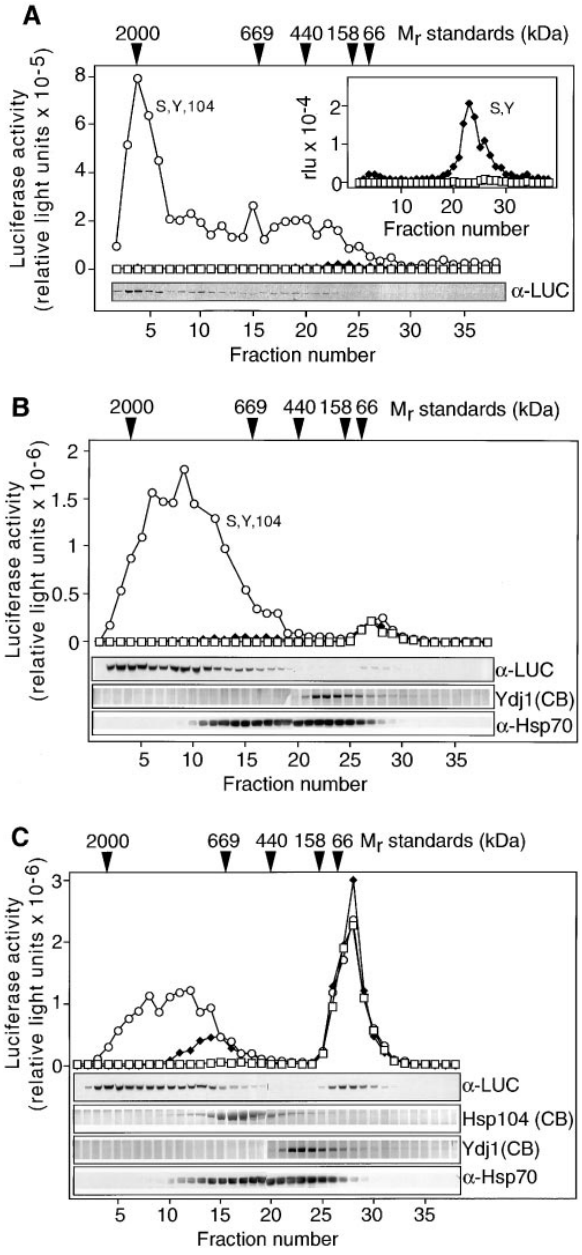

Figure 6. Refolding of Preformed Aggregates of Denatured Luciferase

(A) Denatured luciferase was diluted to 1 μM into ice-cold buffer. After 30 min, particulate material was removed by filtration and the remaining luciferase was fractionated by gel filtration at 4°C. The elution profile of luciferase was determined by Western blotting (α-LUC). For refolding, ice-cold column fractions were supplemented with 5 mM ATP without chaperones (open squares), with 1 μM Ssa1 and Ydj1 (filled diamonds), or with 1 μM Ssa1, Ydj1, and Hsp104 (open circles), and then incubated at 25°C. Luciferase activity was determined after 90 min of refolding. Inset displays refolding data for no chaperone control and Ssa1 and Ydj1 on an expanded vertical axis. Arrowheads indicate elution positions of molecular size standards.

(B) Denatured luciferase (1 μM) was diluted at 25°C into 5 mM ATP and 5 μM each of Ssa1 and Ydj1. After 30 min the reaction was cooled on ice, particulates were removed by filtration, and the remaining soluble protein was fractionated by gel filtration. Elution profile of Ydj1 was detected by Coomassie blue staining (CB). Ssa1 reaction containing all three proteins, which was then fractionated before refolding was complete. Some native luciferase had formed prior to fractionation (Figure 6C). However, the nonnative luciferase remaining was in the form of large aggregates. Hsp104 did not change the nature of the refolding requirements of such aggregates during their formation—after fractionation, all three proteins were still required for refolding (note that only the luciferase in fractions that coeluted with hexameric Hsp104 ~600 kDa was refolded upon addition of Ssa1 and Ydj1 alone). Thus, not only does Hsp104 fail to block aggregation by itself, but it also does not further reduce the aggregation or alter the refolding requirements of nonnative luciferase that has aggregated in the presence of Ssa1 and Ydj1. These experiments support the hypothesis that the primary function of Hsp104 in thermotolerance is to help refold proteins that have already aggregated.

Hsp104-Mediated Refolding with Other Chaperones

Two things suggest that Hsp104-mediated refolding of proteins from an aggregated state may involve the formation of a larger chaperone complex. First, we detected a physical interaction between Hsp104 and Ydj1 (Figure 3A). Since Ydj1 interacts with *SSA*-encoded Hsp70, it might recruit Hsp70 into a complex with Hsp104. Second, Hsp104-mediated refolding is tightly linked to the presence of the Hsp70/40 cofactors and cannot be separated into sequential reactions. Specifically, Hsp104 does not generate products that could be reisolated and subsequently refolded by Ssa1 and Ydj1 or vice versa. Nonetheless, under refolding conditions, any complexes that might form between the chaperones and their substrate, or among the chaperones themselves, are not sufficiently stable to survive fractionation, perhaps reflecting the dynamic nature of their interactions.

To investigate the specificity of Hsp104/cochaperone interactions, we examined Hsp104's ability to function with other chaperones. By delaying the addition of Hsp104, we assessed the ability of chaperones to stabilize the substrate in a retrievable form and also determined whether they could cooperate with Hsp104 in refolding. Rabbit reticulocyte lysate, a rich source of molecular chaperones (Frydman et al., 1994; Shumacher et al., 1996), stabilized nonnative luciferase and also functioned in Hsp104-dependent refolding (Figure 7A). In contrast, *E. coli* DnaK, DnaJ, and GrpE were capable of stabilizing nonnative luciferase in a refoldable state, but refolding required addition of Ssa1 and Ydj1 together with Hsp104 (Figure 7B). These results suggest that the

(α-Hsp70) and luciferase (α-LUC) were detected by western blotting. For refolding, ice-cold column fractions were supplemented with 5 mM ATP without chaperones (open squares), with 1 μM Ssa1 and Ydj1 (filled diamonds), or with 1 μM Ssa1, Ydj1, and Hsp104 (open circles). Luciferase activity was determined after 90 min of refolding at 25°C.

(C) The experiment described in (B) was repeated with Hsp104 (5 μM) in the initial reaction. The elution profile of Hsp104 was determined by Coomassie blue-staining (CB). Note that refolding by Ssa1 and Ydj1 in fractions 10 to 20 is enhanced by Hsp104 present in column fractions.

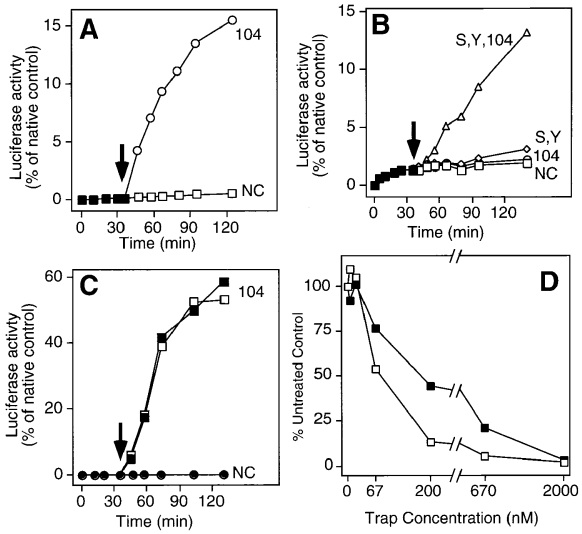

Figure 7. Interactions of Other Chaperones with Hsp104 in Refolding

All reactions contained 20 nM unfolded luciferase and 5 mM ATP.

(A) Refolding of luciferase was monitored in rabbit reticulocyte lysate (diluted ~15-fold in refolding buffer) alone (filled squares). Either buffer (B; open squares) or 1 μM Hsp104 (104; open circles) was added after 35 min (arrow).

(B) Refolding of luciferase was monitored in a reaction (filled squares) containing DnaK (1 μM), DnaJ (0.7 μM), and GrpE (0.5 μM). After 35 min the reaction was supplemented with buffer (B; open squares), 1 μM Hsp104 (104; open circles), 1 μM each of Ssa1 and Ydj1 (S,Y; diamonds), or 1 μM each of all three yeast chaperones (104,S,Y; triangles).

(C) Luciferase refolding was monitored in a reaction containing 1 μM each of the yeast chaperones Ssa1 and Ydj1 (open circles), or in a hybrid chaperone system composed of 1 μM Ssa1 and 0.67 μM DnaJ (filled circles). After 35 min reactions were supplemented with buffer (B; open and filled circles) or 1 μM Hsp (Ydj1/Ssa1+104, filled squares; DnaJ/Ssa1+104, open squares).

(D) Unfolded luciferase (100 nM) was refolded in the presence of the indicated concentrations of mutant GroEL 14-mers together with either 1 μM each of Ssa1 and Ydj1 (open squares), or with 1 μM each of all three yeast proteins (filled squares). Yield of refolded luciferase was measured after 90 min and normalized to the yield of identical reactions without the GroEL "trap" (100%).

prokaryotic proteins do not interface correctly with Hsp104 in the refolding reaction. Interestingly, Ydj1 is not the key factor in determining this specificity. Hsp104-mediated refolding was efficient whether Ssa1 was paired with Ydj1 or its prokaryotic counterpart DnaJ (Figure 7C).

Monomeric, nonnative intermediates that are brought into the bulk solution by the action of Hsp104 might have a chance of folding spontaneously or interacting with other chaperones. However, Hsp104 by itself refolds protein with extremely weak efficiency (Figure 2A). Furthermore, Hsp104 does not produce intermediates that are substrates for refolding by the prokaryotic DnaJ/DnaK/GrpE system (Figure 7B). These dual observations suggest that soluble monomeric intermediates that are independently capable of refolding are produced only at the end of a chaperone-assisted pathway that begins with aggregated Hsp104 targets and requires eukaryotic Hsp70.

To further probe the pathway of intermediates in refolding from aggregates, we examined the sensitivity of yield of refolded protein to the introduction of a GroEL "trap" molecule (Bruston et al., 1996). The chaperonin GroEL binds monomeric nonnative proteins within its central cavity and normally releases them in a reaction cycle driven by ATP hydrolysis and exchange. The GroEL "trap" molecule (337,349) binds but does not efficiently release substrate, even in the presence of ATP. At high concentrations, the trap should immediately bind all the nonnative luciferase. As expected, no nonnative luciferase can be refolded under these conditions (Figure 7D). At intermediate concentrations, the yield from refolding reactions containing Ssa1 and Ydj1 was sensitive to the "trap" molecule, presumably because the unaggregated substrates that are refoldable by the Hsp70 system can also be bound by GroEL (Buchberger et al., 1996). At the same concentrations, reactions containing Hsp104 are less sensitive to the "trap" molecule. It is possible that the yeast chaperones outcompete the "trap" for initial binding of luciferase only when Hsp104 is present. It seems more likely that the reduced sensitivity of Hsp104-mediated refolding to the "trap" protein is due to the efficient, and perhaps partially sequestered, transfer of aggregated substrates (which are inaccessible to the "trap") via Hsp104 to the Ydj1/Ssa1 chaperones, which can release them in a folded or committed-to-refolding form.

Refolding of β-Galactosidase

To test the generality of Hsp104 action on aggregated substrates, we used β-galactosidase as an alternative model. Firefly luciferase is active as a monomer and has a complex folding pattern with a number of native tertiary contacts formed by elements that are distant from each other in the protein's primary sequence (Conti et al., 1996). In contrast, β-galactosidase is active only as a tetramer, and each subunit has five domains whose structural elements are composed of contiguous sequence elements (Jacobson et al., 1994). The proteins also have distinct chaperone-related forward folding properties in vivo (Nathan et al., 1997). In unfractionated experiments, denatured β-galactosidase at low concentration (20 nM) showed little dependence on Hsp104 for refolding. At higher concentrations (≥200 nM), where aggregation was favored, refolding was more dependent on Hsp104 (data not shown).

To investigate more rigorously the relationship between the degree of aggregation and the dependence on Hsp104 for refolding, the experiment illustrated in Figure 6A was repeated using β-galactosidase aggregated in ice-cold buffer in place of firefly luciferase (Figure 8). The absence of any enzyme activity eluting at ~460 kDa, the size of active β-galactosidase tetramers, indicated that no active enzyme was formed prior to fractionation. After incubation at the refolding temperature in the absence of chaperones, some of the nonnative monomer (115 kDa) refolded (or assembled into active tetramers) spontaneously. Ssa1 and Ydj1 alone were able to refold substantial amounts of β-galactosidase that fractionated with an apparent molecular weight of up to ~800 kDa. A greater yield was obtained from these fractions when Hsp104 was added. As was the case for firefly luciferase, larger β-galactosidase aggregates were highly dependent on Hsp104 for refolding.

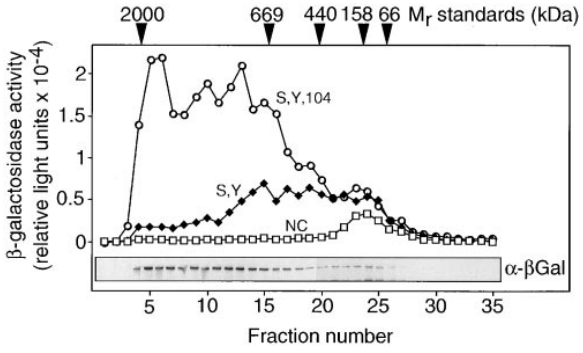

Figure 8. Refolding of Previously Aggregated β-Galactosidase

Denatured β-galactosidase was diluted to 0.8 μM (monomer concentration) into ice-cold buffer. After 30 min, particulate material was removed by filtration through a precooled 0.22 μm filter, and the remaining β-galactosidase was fractionated by gel filtration at 4°C. The elution profile of β-galactosidase was determined by Western blotting (α-βGal). For refolding, ice-cold column fractions were supplemented with 5 mM ATP without chaperones (open squares), with 1 μM Ssa1 and Ydj1 (diamonds), or with 1 μM Ssa1, Ydj1, and Hsp104 (open circles), and then incubated at 25°C. β-galactosidase activity was determined after 90 min of refolding.

Discussion

Hsp104 is not only the most potent thermotolerance protein known but also has the capacity to modulate the aggregation state of both yeast (Patino et al., 1996) and mammalian prion precursors (DebBurman et al., 1997). Elucidation of the mechanism of Hsp104 action will open a new window on our understanding of the dynamics of protein folding and misfolding in vivo and in vitro. We have taken a major step toward this goal by reconstituting a chaperone-facilitated refolding system that requires Hsp104 and specifically utilizes aggregated, trapped nonnative protein as the substrate. Substrates in the in vitro refolding reactions populate a kinetically unstable continuum of aggregation states, including those that are monomeric and refold in a chaperone-independent manner, those that are slightly aggregated and can be at least partially refolded by conventional chaperones, those that are further aggregated and require Hsp104, and those that are highly aggregated and largely irretrievable. Because the functions of DnaK/DnaJ/GrpE in refolding are similar to those of Hsp70/Ydj1, the specificity of Hsp104's requirement for eukaryotic versus prokaryotic Hsp70 suggests that the pathway that intermediates traverse following remobilization from the aggregates involves efficient passage of substrates from Hsp104 to the eukaryotic Hsp70 chaperone complex. This suggestion is supported by the effects of the GroEL "trap." The synergy imparted by the interaction between *SSA*-encoded Hsp70, Ydj1, and Hsp104 suggests that they form a novel chaperone machine that helps cells to recover from exposure to extreme conditions by directly participating in the reversal of stress-induced protein aggregation and the reactivation of critical cellular targets.

In yeast, Hsp104 is not essential, even at temperatures at the upper limit for growth. Hsp104 enhances survival by only 10-fold when cells are exposed to moderately high temperatures (e.g., 44°C) where long exposures are required to lose viability (Sanchez et al., 1992). At extreme temperatures (e.g., ≥50°C), where viability is lost very rapidly, Hsp104 enhances survival from 100- to >1000-fold. Thus, Hsp104 is most important when the rate of protein unfolding exceeds the capacity of other chaperones to prevent the accumulation of aggregates. During recovery from such severe conditions, Hsp104 promotes disaggregation of heat-damaged proteins (Parsell et al., 1994b). Here we've shown that Hsp104 orchestrates this process by working directly on previously aggregated protein, enhancing refolding by >500-fold. We believe this reconstitutes protein rescue in vitro that approximates the quantitative effects of Hsp104 function in thermotolerance. Our current model for Hsp104 function is that Hsp104's hexameric structure provides multiple sites for polypeptide substrate binding. Upon ATP binding and hydrolysis, conformational changes in Hsp104 perforce change the positions of bound substrates relative to each other—in effect, Hsp104 acts as a molecular "crowbar." The inability of Hsp104 to cooperate with *E. coli* Hsp70 (DnaK) provides a molecular explanation for the inability of Hsp104 to complement the thermotolerance defect of *E. coli clpB* mutants (K. Uno and S. L., unpublished observation) and the failure of ClpB to substitute for Hsp104 in yeast thermotolerance (Parsell et al., 1993). However, plant-derived Hsp104 homologs do provide thermotolerance in yeast (Lee et al., 1994; Schirmer et al., 1994). These observations, combined with the ability of Hsp104 to function with rabbit reticulocyte lysate, predict that eukaryotic HSP100s will correctly interface with other eukaryotic Hsp70 chaperones. Similar constraints may apply to prokaryotic homologs. Such observations provide a guide for the use of HSP100 proteins in engineering stress tolerance and expression systems.

The Hsp70 chaperone system plays a critical, dual role in Hsp104-mediated refolding. First, because we do not detect refolding by Hsp104 alone, it is probable that Ssa1 and Ydj1 provide the primary refolding function even in Hsp104-dependent reactions. Second, at higher temperatures, the Ssa1/Ydj1 chaperone system reduces the partitioning of nonnative proteins to an irretrievably aggregated state. A functional relationship between Hsp104 and the *SSA*-encoded Hsp70s was implied in previous work. For example, the reactivation of unfractionated heat-damaged splicing extracts required either Hsp104 or Hsp70 but was more efficient with both proteins (Vogel et al., 1995). At the genetic level, when Hsp70 accumulation is reduced, protein aggregates are evident even at normal temperatures (Lindquist et al., 1995) and Hsp104 becomes essential for normal growth (Sanchez et al., 1993). In the absence of Hsp104 (where cells are more dependent on preventing rather than reversing aggregation), Hsp70 overexpression partially restores the ability of cells to survive extreme stress (Sanchez et al., 1993). The fresh insights obtained by taking a comprehensive approach to reconstructing Hsp104 function in vitro provide a mechanistic rationale for previously observed interactions.

Release of nonnative proteins from aggregates might alone be sufficient to promote refolding. Why then do

we observe Hsp104-mediated refolding only in concert with other chaperones? One possibility is that Ydj1 and Ssa1 are required to direct Hsp104 to its targets. However, the observations that certain peptides and proteins stimulate the ATPase activity of Hsp104 and that mixtures of Hsp104 with Sup35 or PrP undergo dramatic shifts in CD spectra (Schirmer and Lindquist, 1997) argue that at least some targets directly interact with Hsp104. A more plausible hypothesis is that Hsp104 transiently disrupts noncovalent intermolecular contacts between aggregated species. By analogy to *E. coli* ClpA and ClpX, proteins that remodel specific oligomeric substrates, making them more accessible to a protease partner complex, we propose that Hsp104 remodels aggregates of nonnative proteins, making them more accessible to other chaperones. Furthermore, Hsp104 targets may be channeled to the Hsp70 chaperone system through transient physical interactions. In the densely crowded macromolecular environment of the cell, premature exposure of heat-damaged proteins in the process of being refolded might lead to reaggregation and limit the productivity of the rescue pathway. Thus, there is an apparent logic to the close cooperation of Hsp104 with the Hsp70 chaperone machine.

As with ClpA and ClpX, whose net effects on substrates are different in the absence of ClpP (Horwich, 1995), the effect of Hsp104 on its targets may be quite different in the absence of its chaperone cofactors. By reexposing aggregation-prone surfaces, Hsp104 has the potential to promote rather than reverse aggregation. In the case of Sup35, a protein that undergoes ordered, self-seeded assembly into amyloid fibers in vitro (Glover et al., 1997), the maintenance and inheritance of the aggregated form in vivo requires a moderate level of Hsp104 (Chernoff et al., 1995). We have proposed that when present at its normally low constitutive level, Hsp104 partially unfolds Sup35 or disassembles Sup35/Sup45 heterodimers, thereby overcoming conformational or kinetic barriers to the formation of ordered Sup35 aggregates (Patino et al., 1996). Alternatively, Hsp104 may dissociate large, preformed Sup35 aggregates into smaller, aggregation-prone particles that seed the aggregation of newly synthesized Sup35 more efficiently. Intriguingly, Hsp104 acting alone also promotes the in vitro conversion of the cellular form of PrP to an aggregated, protease-resistant form with characteristics of PrP^{Sc} in reactions that are otherwise inefficiently seeded by PrP^{Sc} (DebBurman et al., 1997). Reconstitution of Hsp104's role in amyloid formation, as we have done in refolding, should yield insight into these remarkable ordered aggregation phenomena.

Experimental Procedures

Proteins

Hsp104 was purified as previously described (Parsell et al., 1994a). The Ydj1 coding sequence was subcloned into pJC25 (from Joachim Clos) and expressed in *E. coli* strain BL21[DE3]pAP*lacI*^{q} (from Olivier Fayet). Cells were grown in Circlegrow (BIO 101) at 37°C to an OD_{600} of ~0.8 and induced for 2 hr with 1 mM IPTG. Ydj1 was purified as previously described (Cyr et al., 1992).For SSa1 overexpression, protease-deficient yeast strain BJ5457 (Jones, 1991) was cotransformed with pG-N795 (Schena et al., 1991) and pUG2-*SSA1* (from Elizabeth Craig). Cells were grown and induced as described for Hsp104 expression. Ssa1-enriched Hsp70 was purified as described (Ziegelhoffer et al., 1995). His-tagged Hsp104 (H10–104) and its mutant derivatives (H10-K218T and H10-K620T) were expressed and purified as described elsewhere (Schirmer et al., 1998). DnaK, DnaJ, and GrpE were purchased from Stressgen. GroEL (wt) and the GroEL "trap" were provided by Arthur Horwich. Rabbit reticulocyte lysate (Green Hectares) was desalted into refolding buffer prior to use. Protein concentrations were determined by a dye-binding assay (BioRad) using BSA as a standard. Molarity of proteins refers to the concentration of monomer except where otherwise indicated.

Preparation of Yeast Lysates

Cells from stationary phase yeast cultures were disrupted with glass beads in 25 mM HEPES-KOH (pH 7.6), 150 mM KOAc, 10 mM $Mg(OAc)_2$, 10 mM DTT (RFB^{lys}) supplemented with 1 mM each of PMSF and benzamidine, 2 $\mu g \cdot ml^{-1}$ each of aprotinin, pepstatin A, and leupeptin. The lysate was clarified by centrifugation at 100,000 × g for 30 min at 4°C and desalted into RFB^{lys}/1 mM PMSF by gel filtration on Sephadex G-25 (Pharmacia) immediately before use in refolding assays. For analysis of Hsp104 function in refolding, the yeast strain BJ5457 carrying a disruption of *HSP104* was used (A798). WT lysate was derived from YPH499 (Sikorski and Hieter, 1989). Hsp104-deficient lysate was derived from YPH499 carrying an *hsp104*::*LEU2* disruption (A1721 made by Y. Kimura). Ydj1-deficient lysate was obtained from a segregant of YPH501 carrying a *ydj1*::*TRP1* disruption (A2217 made by Y. Kimura). Lysate deficient in both proteins was obtained from a segregant (A2889) of a diploid derived from mating A1721 with A2217.

Protein Affinity Chromatography

Two hundred microliters packed Ni^{2+}-NTA resin (Qiagen) was incubated together with 1 mg H10–104 for 15 min in 25 mM HEPES-KOH (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 1.4 mM β-mercaptoethanol (buffer B). The resin was washed to remove unbound protein. The 100,000 × g supernatant of lysate prepared from A798 cells was desalted into buffer B containing a cocktail of protease inhibitors. Five milligrams of lysate was incubated together with resin for 10 min at room temperature, then washed sequentially on-column with 10 volumes each of buffer B, buffer B with 1M NaCl, and buffer B with 20 mM imidazole, and finally eluted with buffer B containing 200 mM imidazole. Nonspecific binding was determined using resin without H10–104 bound.

Ion Exchange Fractionation of Lysate

Lysate was prepared from protease-deficient strain BJ5457 and desalted into 20 mM TRIS-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT (Q buffer) containing 0.5 mM PMSF. Twenty milligrams (5 ml) of lysate was fractionated on a 1 ml Resource Q anion exchange column with a linear gradient of 0–750 mM KCl in 20 ml of Q buffer collecting 1.25 ml fractions.

Western Blotting

Rabbit anti-Hsp26, rabbit anti-Hsc/p82, and rat anti-Hsp70 monoclonal antibody were produced in this laboratory. Rabbit anti-β-galactosidase (Cappel) and rabbit anti-firefly luciferase (Promega) were purchased from commercial sources. Rabbit anti-Ssa (#343) and anti-Ssb (#344), rabbit anti-Ydj1, and rabbit anti-Sis1 were supplied by John Halladay, Avrom Caplan, and Kim Arndt, respectively. Immune complexes were visualized by enhanced chemiluminescence detection (Amersham).

Refolding Assays

Refolding procedures were modeled after several previously reported methods (Levy et al., 1995; Freeman and Morimoto, 1996; Shumacher et al., 1996). The most significant deviation from published methods was the use of urea as an unfolding agent. The ATPase activity of Hsp104 was inhibited by the low residual concentration of guanidinium-HCl present in refolding reactions following addition of the unfolded substrate (J. R. G. and S. L., unpublished observation). Firefly luciferase (Sigma) was dissolved in 1 M glycylglycine (pH 7.4), diluted into 4–8 M urea made up in refolding buffer, and incubated at 30°C for 30 min. In some experiments, recombinant luciferase (Promega) was used with essentially the same results.

For refolding in lysates, the unfolded protein was diluted 100-fold (0.5 μl into a total reaction volume of 50 μl) into buffer consisting of RFB^{lys}, which, unless otherwise specified, contained 100 mg lysate, 5 mM ATP, and an ATP-regenerating system consisting of 20 mM phosphocreatine (Sigma), 0.5 mg creatine phosphokinase (Sigma), and chaperones. Ion exchange fractions were desalted into RFB^{lys} prior to use in refolding assays. For refolding using purified chaperones, the buffer was 25 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT (RFB) supplemented with 0.1 mg · ml^{-1} BSA to stabilize activity of refolded enzyme. The ATP-regenerating system was omitted. At indicated times postdilution, duplicate 1 μl aliquots were removed and enzyme activity determined in 50 μl of luciferase substrate solution (Promega). Light emission was measured in an OPTICOMP 1 luminometer (MGM Instruments) and accumulated over 5 s. When necessary, dilutions were made in RFB/0.1 mg · ml^{-1} BSA immediately prior to measurement. One hundred percent native activity was determined by performing identical dilutions but omitting the urea unfolding step. β-galactosidase activity was determined in duplicate 1 μl aliquots of refolding reactions by addition of 67 μl of Galacton (TROPIX) followed by incubation at ambient temperature for exactly 10 min. Light emission was measured in a luminometer for 10 s following automated injection of 100 μl Light Emission Accelerator (TROPIX).

Turbidity

Luciferase (30 μM) was unfolded in 4 M urea/RFB for 30 min at 30°C and diluted 50-fold into RFB at room temperature with specified additions. Turbidity was measured monitoring absorbance at 320 nm at ambient temperature.

Gel Filtration

Luciferase (50 μM) was unfolded in 4 M urea for 30 min at 30°C and diluted 50-fold into ice-cold RFB/0.1 mg · ml^{-1} BSA, or at 25°C into the same buffer with the additions indicated in figure legends. β-galactosidase (80 μM) was unfolded in 8 M urea/RFB for 30 min at 30°C and diluted 100-fold into cold buffer. After 30 min incubation, reactions were cooled on ice and filtered through precooled 0.22 μm filters (Millex GV_4, Millipore). Then, 0.25 ml was injected onto a 1 × 30 cm Superose 6 column (Pharmacia) equilibrated in RFB/0.1 mg · ml^{-1} BSA at 4°C. The column was developed at a flow rate of 0.4 ml · min^{-1}. Fractions (0.4 ml) were collected beginning 15 min postinjection. Twenty-five microliters of each fraction was analyzed by SDS-PAGE. The gel filtration size standards were blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and bovine serum albumin (66 kDa).

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