THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 28, Issue of July 14, pp. 21107–21113, 2000

Printed in U.S.A.

Size-dependent Disaggregation of Stable Protein Aggregates by the DnaK Chaperone Machinery*

Received for publication, February 16, 2000, and in revised form, April 27, 2000
Published, JBC Papers in Press, May 2, 2000, DOI 10.1074/jbc.M001293200

Sophia Diamant‡, Anat Peres Ben-Zvi‡, Bernd Bukau§, and Pierre Goloubinoff‡¶

From the ‡Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel and the §Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder Strasse 7, D-79104 Freiburg, Germany

Classic *in vitro* studies show that the Hsp70 chaperone system from *Escherichia coli* (DnaK-DnaJ-GrpE, the DnaK system) can bind to proteins, prevent aggregation, and promote the correct refolding of chaperone-bound polypeptides into native proteins. However, little is known about how the DnaK system handles proteins that have already aggregated. In this study, glucose-6-phosphate dehydrogenase was used as a model system to generate stable populations of protein aggregates comprising controlled ranges of particle sizes. The DnaK system recognized the glucose-6-phosphate dehydrogenase aggregates as authentic substrates and specifically solubilized and refolded the protein into a native enzyme. The efficiency of disaggregation by the DnaK system was high with small aggregates, but the efficiency decreased as the size of the aggregates increased. High folding efficiency was restored by either excess DnaK or substoichiometric amounts of the chaperone ClpB. We suggest a mechanism whereby the DnaK system can readily solubilize small aggregates and refold them into active proteins. With large aggregates, however, the binding sites for the DnaK system had to be dynamically exposed with excess DnaK or the catalytic action of ClpB and ATP. Disaggregation by the DnaK machinery in the cell can solubilize early aggregates that formed accidentally during chaperone-assisted protein folding or that escaped the protection of "holding" chaperones during stress.

A network of molecular chaperones in the cell controls the correct folding of nascent and translocating polypeptides, the stability of native proteins under stress, and the refolding of denatured proteins following stress (for reviews see Refs. 1 and 2). Chaperones can specifically recognize and interact with non-native proteins, mostly through hydrophobic interactions with exposed hydrophobic surfaces. Some chaperones such as HtpG, DnaK, DnaJ, GroEL, and IbpB from *Escherichia coli* are also termed "holders" because they can form a binary complex with unstable protein folding intermediates and thus prevent aggregation (for a review see Ref. 3). However, under physiological conditions chaperones such as GroEL in the presence of GroES and ATP or DnaK in the presence of DnaJ, GrpE, and ATP can also act as "folders," which interact in a more dynamic manner with protein folding intermediates and thus maintain proteins within folding pathways that lead to the native structure. Much remains to be understood about the molecular mechanism through which different chaperone systems assist protein folding. Cycles of binding and release that are fueled by ATP and hydrolysis may be a common theme in the mode of action of chaperones that are as structurally and functionally different as DnaK, GroEL, and HtpG (2).

Problems arise during stress when unfolded proteins become highly unstable and escape the protective action of the "holding" reservoir of the chaperone network. Soluble unfolded proteins do not remain in solution waiting for assisted refolding by "folding" chaperones after stress. Rather, unfolded proteins tend to assume an alternatively stable conformation in the form of insoluble aggregates enriched with β-pleated structures and exposed hydrophobic surfaces that remain inactive after stress (4, 5).

In vitro, most chaperone systems are inefficient in actively dissolving protein aggregates. For the DnaK system, only one report mentions that a molar excess of the DnaK system can efficiently reactivate previously heat-aggregated RNA polymerase (6, 7). Negligible amounts of pre-aggregated enzymes such as luciferase are recovered in the presence of a large (100–1000-fold) molar excess of the DnaK or Hsp70 chaperone systems (8–11). In contrast, substoichiometric levels of the DnaK system in the presence of the chaperone ClpB suffice to efficiently solubilize and reactivate a wide array of previously aggregated *E. coli* and model protein substrates (12, 13). ClpB was shown to modify and precondition the nature of large turbid aggregates toward subsequent solubilization and refolding by the DnaK system (12). However, the molecular mechanism by which the DnaK system alone mediates efficient solubilization of protein aggregates and the spectrum of action of the DnaK system with regard to the nature and size of the aggregated substrates remain unclear.

In this study, we address the mechanism by which the DnaK chaperone system achieves solubilization and refolding of protein aggregates that have different degrees of complexity. Using stable forms of aggregates of glucose-6-phosphate dehydrogenase (G6PDH)[1] as model substrates, we have demonstrated that the DnaK system alone can directly interact, disaggregate, and reactivate stably aggregated protein particles. The efficiency of disaggregation decreased as the size of the aggregate increased, but efficient disaggregation was restored by a large molar excess of DnaK or catalytic amounts of ClpB.

MATERIALS AND METHODS

Proteins—Protein purifications were performed according to published procedures: DnaK, DnaJ, and GrpE (14), ClpB (15), GroEL and

* This work was supported in part by grants from the German Israeli Foundation and the Abisch-Frenkel Foundation (to P. G.) and from the Deutsche Forschungsgemeinschaft (to B. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 972-2-6585391; Fax: 972-2-6584425; E-mail: pierre@vms.huji.ac.il.

[1] The abbreviations used are: G6PDH, glucose-6-phosphate dehydrogenase; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid.

GroES (16), and IbpB (17). Rabbit muscle pyruvate kinase and lyophilized G6PDH from *Leuconostoc mesenteroides* were purchased from Sigma. G6PDH was resuspended in 20% glycerol to a final concentration of 300 μM and used as a stock solution. Protein concentrations were determined using the Bio-Rad Bradford assay with bovine serum albumin as a standard. Protein concentrations are always expressed in protomers.

G6PDH Denaturation—Various concentrations of native G6PDH were denatured in three steps: (i) unfolding (5 min at 47 °C in 5 M urea, 20 mM dithiothreitol, and 8% glycerol); (ii) dilution (50-fold dilution in folding buffer (50 mM triethanolamine, pH 7.5, 20 mM $MgAc_2$, 150 mM KCl, 10 mM dithiothreitol, and 3 mM phosphoenolpyruvate) at 47 °C); (iii) stabilization (15 min at 47 °C). Large turbid G6PDH aggregates were formed by a 20 min incubation in folding buffer at 65 °C.

Size-exclusion Chromatography—The oligomeric state and apparent size of the various forms of denatured G6PDH were estimated by size-exclusion chromatography in buffer (50 mM triethanolamine, pH 7.5, 20 mM $MgAc_2$ and 150 mM KCl), using a Superose 6HR10/30 gel filtration column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min. The absorbance was monitored at 280 nm. Apparent molecular weights were estimated by gel filtration standards (Bio-Rad).

Spectroscopy—Spectroscopic measurements were performed in a Perkin-Elmer luminescence spectrometer LS50B. The various forms and concentrations of aggregated G6PDH were incubated in folding buffer with 50 μM 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS, Molecular Probes) for 5 min. bis-ANS fluorescence of G6PDH was measured with excitation at 397 nm and emission at 496 nm. Turbidity was measured in a four-sided quartz cuvette at an excitation and emission wavelength of 550 nm.

Chaperone Activity Assay—Assays were performed in folding buffer in the presence of an ATP-regenerating system (3 mM phosphoenolpyruvate and 20 μg/ml pyruvate kinase), which was active for the concentration chaperones tested over at least 5 h at 30 °C. Unless otherwise specified, concentrations and relative chaperone ratios in the DnaK system remained constant (3.5 μM DnaK, 0.7 μM DnaJ, 0.35 μM GrpE). Refolding of G6PDH was initiated at 30 °C by the addition of 3 mM ATP and pyruvate kinase. Maximal rates of G6PDH refolding were derived from the linear phase of the time curves of recovered enzymatic activity (18). Similar rates and yields of the DnaK-mediated reactivation of aggregated G6PDH were obtained when the DnaK chaperone system, ATP, and pyruvate kinase were added immediately or after a delay of up to 30 min following heat stabilization (data not shown). This demonstrates the high stability of the G6PDH aggregates. In general, chaperones and ATP were added within 4 min after the final heat stabilization step of the G6PDH aggregates.

Enzymatic Assays—The G6PDH activity was measured using the spectroscopic method as described in Ref. 18. The ATPase activity was measured by a colorimetric method for phosphate determination using the phosphorus diagnostic reagent from Sigma (19).

RESULTS

Characterization of Inactive G6PDH Species—To analyze the mechanism by which the DnaK chaperone system specifically solubilizes and refolds populations of stable protein aggregates of varying sizes, we first identified and characterized appropriate substrates for the reaction. When native G6PDH was pretreated with dithiothreitol and 5 M urea at 47 °C for 5 min and then diluted to final concentrations ranging from 0.2 to 2.8 μM in folding buffer, significant levels of spontaneous reactivation occurred at 30 °C. However, spontaneous reactivation was abolished following an additional incubation of 15 min at 47 °C. Only this complex procedure (urea and heat denaturation, dilution, and a final heat stabilization step) was able to reproducibly generate populations of non-turbid yet stable and inactive aggregates with defined size distributions (see below). Incubation for 20 min at 65 °C also yielded stable inactive aggregates that were turbid with undefined high degrees of complexity.

Gel filtration chromatography of the G6PDH forms that were both denatured and heat-stabilized at four different concentrations (0.3–1.5 μM, Fig. 1*a*) showed that the majority of species was resolved in a continuum of soluble small oligomeric particles ranging from 106 kDa (Fig. 1*a*, *peak III*) to 5,000 kDa (*peak II*). Only a minor fraction eluted in the void volume (5–40 $\times$ 10^3 kDa, according to the specifications of Amersham Pharmacia Biotech) (Fig. 1*a*, *peak I*). As the final concentration at which the protein was heat-stabilized was increased, the majority of the protein species in peak II was shifted toward a heavier molecular weight (Fig. 1*a*, *inset*). Although still soluble, more material eluted in the void volume at the expense of lower molecular weight forms (Fig. 1*a*). It should be noted that because aggregated proteins are likely to assume non-globular shapes, it is not possible to estimate the precise oligomeric state of the soluble G6PDH aggregates (4, 5).

The light scattering intensity of the inactive aggregates stabilized at 47 °C was remarkably low; aggregate concentrations of 0.4 and 2 μM scattered light less than 1 and 7%, respectively, when compared with that of the same protein concentrations

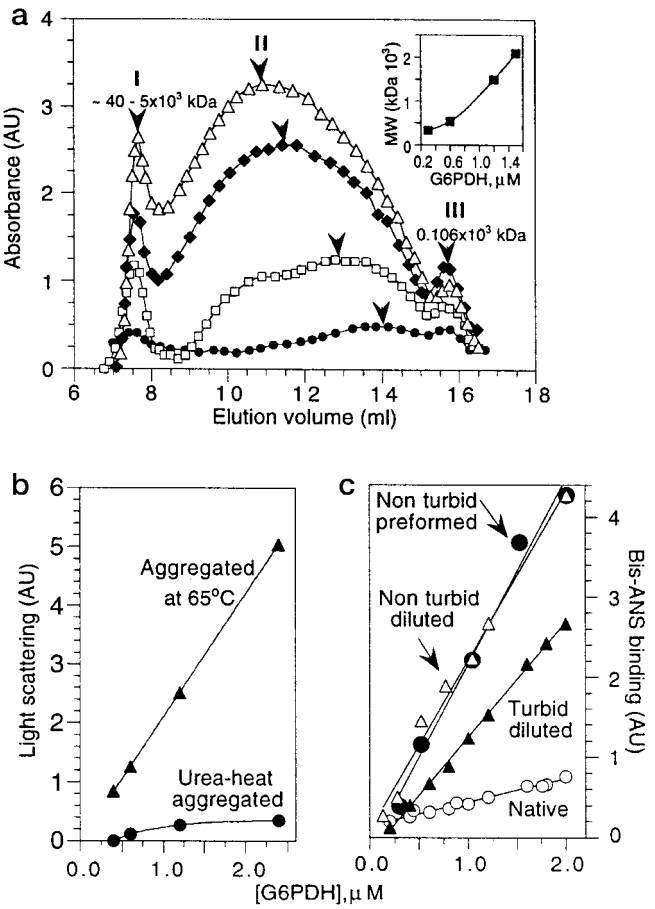

FIG. 1. **Characterization of denatured G6PDH.** *a*, size fractionation of denatured G6PDH aggregates. G6PDH was urea/heat-denatured at 0.3 μM (*filled circles*), 0.6 μM (*open squares*), 1.2 μM (*filled diamonds*), and 1.5 μM (*open triangles*) as described under "Materials and Methods" and fractionated by gel filtration chromatography. *Peak I*, aggregates eluting in the void volume above 5,000 kDa; *peak II*, aggregates below 5,000 kDa but above 106 kDa; *peak III*, inactive 106-kDa G6PDH dimers; *inset*, estimated molecular mass of the most abundant species in peak II (*arrowheads*) as a function of the concentration at which the G6PDH aggregates were stabilized. *b,* turbidity of denatured G6PDH. Light scattering intensity at 550 nm of urea/heat-denatured G6PDH at increasing concentrations (*filled circles*) was compared with heat-denatured G6PDH (65 °C) at 2.5 μM and diluted to the indicated concentrations (*filled triangles*). *c,* hydrophobic exposure of denatured G6PDH. The fluorescence intensity of bis-ANS in the presence of urea/heat-denatured G6PDH at increasing concentrations (*filled circles*) was compared to G6PDH that was urea/heat-denatured at 2.0 μM and then diluted to the indicated concentrations (*open triangles*), to native G6PDH (*open circles*), or to G6PDH that was heat-denatured at 65 °C at 2.0 μM and diluted to the indicated concentrations (*filled triangles*).

denatured at 65 °C (Fig. 1*b*). Moreover, following centrifugation at 16,000 × *g* for 5 min, the majority of the inactive G6PDH remained in solution (data not shown). We conclude that in contrast to the large turbid aggregates formed at 65 °C, the inactive G6PDH species stabilized at 47 °C are virtually non-turbid. As demonstrated by gel filtration chromatography, these species were significantly smaller (consisting of mostly soluble particles) but was nonetheless as stable as large turbid aggregates and did not refold spontaneously (data not shown).

Indicative of increased hydrophobic exposure, non-turbid aggregates bound 7 times more bis-ANS than the native enzyme (Fig. 1*c*) and 1.5 times more bis-ANS than the turbid aggregates. This implies that non-turbid aggregates exposed more hydrophobic residues than turbid aggregates. bis-ANS binding was proportional to the concentration at which the aggregate was initially stabilized or subsequently diluted (Fig. 1*c*).

The Aggregates Are Specific Substrates for the DnaK System—All concentrations of the inactivated G6PDH showed less than 0.1% spontaneous refolding. Moreover, no significant reactivation was observed in the presence of ATP and GroEL/GroES or ClpB or IbpB chaperones (data not shown). However, in the presence of the DnaK system and ATP, a variety of aggregate sizes and concentrations were specifically reactivated after different lag times and at different rates and yields (Fig. 2*a*). This demonstrates that several forms of non-turbid G6PDH aggregates are authentic substrates that can be specifically disaggregated and refolded by the DnaK system although in a manner that depends on the size and concentration of the particles.

GroEL/GroES failed to reactivate even the smallest G6PDH particles (data not shown) confirming that the size limit for DnaK substrates exceeds that of GroEL substrates (>60 kDa) (3, 20). However, we also found that as the size of the aggregates increased, aggregates became increasingly poorer substrates for the DnaK system. The lag time necessary to reactivate the same low concentration of 50 nM G6PDH by the same concentration of DnaK chaperones (3.5 μM) was 24-fold longer with 2.9 μM aggregated G6PDH than with an 8 times lower concentration of substrate (0.36 μM) (Fig. 2*a*, *inset*). Hence, unlike a classic enzymatic reaction in which the velocity of the reaction with a fixed amount of enzyme increases with the concentration of substrate and becomes maximal in the presence of saturating concentrations of substrate (V_{max}), the reactivation rates carried out by the DnaK system and ATP initially increased up to 0.6 μM G6PDH and then rapidly decreased as the concentration and size of the aggregates increased above 0.6 μM (Fig. 2*b*). We found that a 6-fold molar excess of DnaK over the substrate was necessary to obtain optimal folding rates and at least a 3-fold molar excess of DnaK for optimal folding yields. When the DnaK/G6PDH ratio was below 3, the yields rapidly decreased (Fig. 2*b*).

DnaK Interacts with Small Protein Aggregates—In the presence of increasing concentrations of non-turbid aggregates, the rate of ATP hydrolysis with a constant concentration of DnaK, DnaJ, and GrpE showed an activation of up to 2-fold (Fig. 2*b*). This confirms that the small G6PDH aggregates are chaperone substrates that interact with DnaK. The DnaK-ATPase activity initially increased and then decreased with the substrate concentration. Interestingly, the spectrum of the DnaK-ATPase activation matched that of the refolding yields rather than that of the refolding rates (Fig. 2*b*). This indicates that the yields of the reaction were affected only when the interaction between the DnaK system and the aggregated substrate was impaired.

The Refolding Activity of the DnaK System Is Limited by the Size of the Aggregates—We next addressed the relationship between the size of the aggregates and their ability to serve as

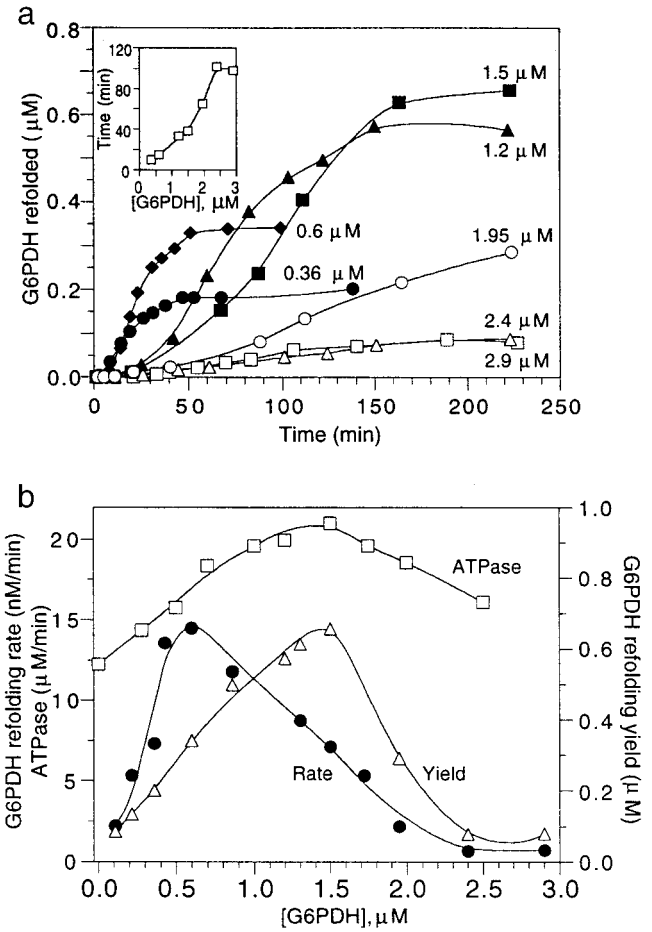

FIG. 2. **Effect of the aggregate concentration on DnaK chaperone-mediated refolding.** The reactivation of preformed aggregates, urea and heat-denatured at the indicated concentrations, was performed in the presence of a fixed amount of DnaK, DnaJ, and GrpE at 3.5, 0.7, and 0.35 μM, respectively, and 3 mM ATP in the presence of an ATP-regenerating system, as described under "Materials and Methods." *a*, time-dependent reactivation of urea/heat-denatured G6PDH at the indicated concentrations. *Inset*, time needed to reactivate 50 nM G6PDH from different aggregate concentrations. *b*, refolding rates (*filled circles*), absolute refolding yields (*open triangles*) of active G6PDH, and rates of DnaK-ATPase (*open squares*) as a function of substrate concentration.

substrates for the DnaK system. Small G6PDH aggregates, initially formed and stabilized at 0.4 μM, were concentrated 20-fold and separated by size-exclusion chromatography (Fig. 3*a*). Noticeably, the subsequent gel filtration pattern was virtually identical to that obtained for the original preparation (data not shown). 87% of the material was recovered demonstrating that the aggregates were soluble and highly stable. Three discrete fractions of soluble aggregates (fractions A, B, and C) were adjusted to the same final concentration (1 μM) and incubated with the same concentration of the components of the DnaK system and ATP (as in Fig. 2*a*). Remarkably, the DnaK system successfully reactivated some 27% of the G6PDH in 2 h although only in the fraction that contained the least complexed aggregates (fraction C). Higher molecular weight aggregates in fractions B and A remained inactive (Fig. 3*b*, *striped columns*). This confirms that the DnaK system efficiently refolds only small protein aggregates. Interestingly, a 5-fold dilution to 200 nM of the aggregates allowed the recovery of some 100 nM G6PDH in fraction C and about half that much in fractions A and B (Fig. 3*b*, *dotted columns*). This suggests that increasing the ratio between the DnaK system and the

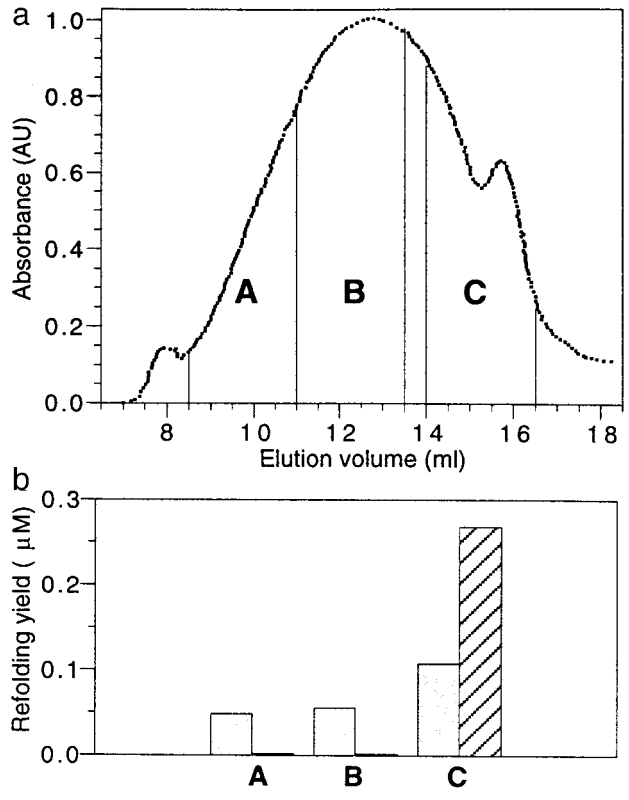

FIG. 3. **Effect of size of aggregates on Dnak-mediated refolding.** *a*, gel filtration profile, urea/heat-denatured G6PDH aggregates were formed and heat-stabilized at 0.4 μM (as in Fig. 1*a*) and then concentrated 50-fold and separated by size-exclusion chromatography. Three fractions of soluble aggregates were collected (*A*, 8.5–11 ml; *B*, 11–13.5 ml; and *C*, 14–16.5 ml). *b*, refolding activity. Fractions *A*, *B*, and *C* were adjusted to a final concentration of 1.0 μM (*striped column*) or 0.2 μM (*dotted column*) and incubated 2 h with DnaK, DnaJ, and GrpE at 3.5, 0.7, and 0.35 μM, respectively, and 3 mM ATP (as described in the Fig. 2 legend).

substrates can compensate for the decreased ability of the DnaK system to recognize, disaggregate, and reactivate large protein aggregates.

The Chaperone/Substrate Ratio Determines the Refolding Efficiency of Large Aggregates—We specifically addressed the role of the ratio between DnaK and large aggregates on the efficiency of the chaperone system. The refolding activity was measured with two types of G6PDH aggregates: small aggregates generated at 0.4 μM that were subsequently concentrated to 2 μM or large non-turbid aggregates that were directly generated at 2 μM. The yield of chaperone-mediated reactivation of small aggregates (formed at 0.4 μM and concentrated thereafter to 1 μM) was 2 times higher than with the same amount (1 μM) of large aggregates formed at 2 μM and diluted thereafter (Fig. 4*a*). However, the same concentration of chaperone became equally efficient at reactivating a 5-fold smaller amount (0.2 μM) of aggregated G6PDH regardless of the particle size (Fig. 4*b*).

Gel filtration confirmed that neither concentrated small aggregates (0.4 μM) nor diluted large aggregates (2 μM) changed oligomeric state during concentration or dilution (Fig. 4, *a* and *b*, *insets*) indicating that both oligomeric structure and the concentration of the aggregate determine the efficiency of the chaperone system. The dependence of the chaperone efficiency on the size and concentration of the aggregate was further addressed over a wide range of concentrations (0.05–2.0 μM) of the small and large types of aggregates described above (Fig. 4*c*). At concentrations up to 0.6 μM G6PDH (a DnaK/G6PDH ratio of >6), the reaction yield was independent of the oligomeric state of the aggregate, but when the substrate concentration exceeded 0.6 μM (a DnaK/G6PDH of <6) the reaction yields strongly decreased. The decrease of the chaperone efficiency was more dramatic with large aggregates than with small aggregates. At 2 μM substrate, the reactivation yields of the reaction with large aggregates was 4 times lower than with small aggregates. This suggests that productive refolding requires concomitant interactions of several chaperone molecules with the same polypeptide within a large aggregate.

Higher DnaK Concentrations Compensate for Reduced Refolding Activity—We further addressed the possibility that limiting DnaK concentrations account for the observed low chaperone efficiency with high aggregate concentrations. The low refolding rates and yields of the reaction of large aggregates (2 μM) were dramatically improved when the DnaK concentration was increased by a factor of 3 from 3.5 to 10 μM (the DnaJ and GrpE remained unchanged). A similar effect was observed when 6.5 μM DnaK was supplemented 150 min after initiation of poorly productive refolding to the initial 3.5 μM DnaK (Fig. 5*a*). This indicates that the ratio between DnaK and the substrate determines the effectiveness of the interaction of the DnaK system with large aggregates. Refolding was similarly improved when the concentration of all three components of the DnaK system was proportionally increased (see below) indicating that the effect primarily depends on the DnaK concentration relative to the substrate rather than on the ratio between DnaK and its co-chaperones, DnaJ and GrpE.

ClpB Can Substitute for High DnaK Concentrations—When inefficient refolding of high molecular weight aggregates (2 μM) with 3.5 μM DnaK (and 0.7 μM DnaJ and 0.35 μM GrpE) was tested in the presence of a substoichiometric level of the ClpB chaperone (0.5 μM), both rates and yields of reactivation were dramatically increased (Fig. 5*b*). The effect of 0.5 μM ClpB concentrations was similar to that of 10 μM DnaK.

In contrast to non-turbid G6PDH particles, a low concentration of large turbid particles (0.4 μM) formed at 65 °C was not reactivated by 3.5 μM DnaK and was only poorly reactivated by 10 μM DnaK (Fig. 5*c*). Only in the presence of 0.5 μM ClpB and 3.5 μM DnaK (and 0.7 μM DnaJ and 0.35 μM GrpE) were the turbid particles reactivated 8 times faster, reaching yields 7 times higher than in the presence of 10 μM DnaK without ClpB. Thus, ClpB appears to render the large turbid aggregates much more compatible for subsequent disaggregation by the DnaK system. Moreover, ClpB significantly reduced the amount of DnaK needed for the disaggregation of non-turbid aggregates and was absolutely essential for the disaggregation of large turbid aggregates.

We tested the ability of 3.5 μM DnaK (and its co-chaperones) to refold increasing concentrations and sizes of aggregate particles with or without ClpB (Fig. 5*d*). The presence of ClpB nearly doubled the recovery of the small aggregates. Remarkably, the ClpB was most effective at alleviating ineffective refolding of large non-turbid aggregates at concentrations of up to 2.9 μM by the DnaK system alone. In the case of the large aggregates, the refolding yields were 28-fold higher than without ClpB (Fig. 5*d*, *inset*). This demonstrates that in the presence of substoichiometric amounts of ClpB, the yields of DnaK-mediated disaggregation and reactivation become equally optimal with all forms of aggregates from small non-turbid to infinitely large turbid aggregates. Thus, confirming initial observations on large aggregates of malate dehydrogenase (12), ClpB alleviates the dependence of the DnaK chaperone machinery on the size and of the aggregated substrates.

The effect of ClpB was confirmed when the refolding yields were analyzed in the presence of increasing concentrations of

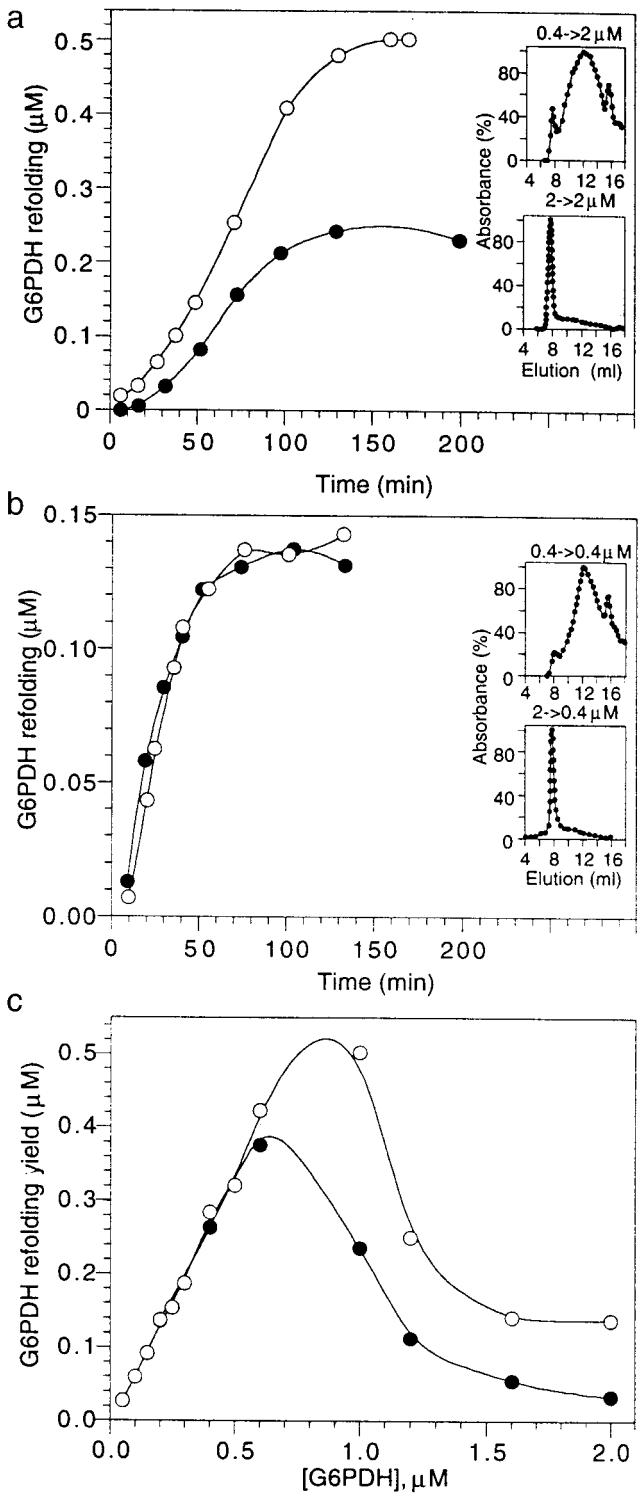

FIG. 4. **Effect of particle concentration and size on DnaK-mediated refolding.** *a*, reactivation of 1.0 μM small particles initially prepared at 0.4 μM and concentrated thereafter (*open circles*) or 1.0 μM large aggregates initially prepared at 2 μM and diluted twice thereafter (*filled circles*) with DnaK, DnaJ, and GrpE (3.5, 0.7, and 0.35 μM, respectively). *Upper inset*, gel filtration elution profile of the 0.4 to >2 μM sample after concentration; *lower inset*, elution profile of the sample initially prepared at 2 μM. *b*, reactivation by the DnaK system of 0.2 μM small particles (*open circles*) or 0.2 μM large particles (*filled circles*) as shown in *a*. *Upper inset*, the elution profile of the sample initially formed at 0.4 μM; *lower inset*, the elution profile of 5× dilution of the sample initially stabilized at 2 μM (2 to >0.4 μM). Elution profiles were expressed in percent of the maximal signal. *c*, reactivation by DnaK, DnaJ, and GrpE (3.5, 0.7, and 0.35 μM, respectively) of various concentrations (0.05–2 μM) of small (*open circles*) or large (*filled circles*) aggregates as shown in *a*.

DnaK (and proportionally of DnaJ and GrpE), without or with ClpB (Fig. 5*e*). Without ClpB, half of the maximal refolding was reached in the presence of about a 4-fold molar excess of DnaK over G6PDH, whereas a near equimolar amount of DnaK sufficed in the presence of ClpB. This can be interpreted as if ClpB increased the apparent affinity of DnaK for the aggregates by increasing the ability of DnaK molecules to interact with the aggregates.

DISCUSSION

During the denaturation of a native protein, unstable folding intermediates that escape chaperone binding may reach an alternatively stable state of inactive insoluble aggregates (5). Stable aggregates remain refractory to subsequent attempts of *in vitro* reactivation by most individual chaperone systems such as GroELS or DnaK-DnaJ-GrpE (8, 9, 10, 17, 20). Only in the specific case of heat-inactivated RNA polymerase was the DnaK chaperone system from *E. coli* and ATP able to mediate efficient disaggregation and reactivation, an exceptional activity that was attributed to an undefined "mild" nature of the aggregate (6, 7).

To better characterize the nature of the protein aggregates that can effectively serve as chaperone substrates and be specifically solubilized by the DnaK system, we developed a method to generate various populations of stable aggregates of G6PDH with a controlled spectrum of particle sizes. These range from small soluble non-turbid aggregates to large yet mostly soluble non-turbid aggregates to even larger and insoluble turbid aggregates. The G6PDH aggregates were stable both in their oligomeric structure and in their ability to serve as chaperone substrates. They did not refold spontaneously. Size-exclusion chromatography indicated a complexity that was unaffected by dilution or concentration. Similar refolding efficiencies were obtained when chaperones were added immediately or following hours of delay. bis-ANS binding was strictly proportional to the concentration of the protein. Hence, the general hydrophobic exposure of the aggregate surfaces was unaffected by the complexity and concentration of the aggregate. Because increasingly larger globular particles display decreasing surface/volume ratios, a constant ratio suggests that G6PDH aggregates are mostly elongated filaments typical of many protein aggregates (4, 5).

Remarkably, the large turbid aggregates formed at 65 °C exposed fewer hydrophobic residues, thus suggesting more collapsed and compact structures. Because hydrophobic residues are core elements of the DnaK and DnaJ binding sites (21), we expected that fewer chaperone molecules would be able to interact with insoluble turbid aggregates than with soluble non-turbid aggregates. This was confirmed by substrate-induced activation of the DnaK ATPase. Whereas DnaK strongly interacted with small non-turbid aggregates, interaction decreased with large non-turbid aggregates and was negligible with very large turbid aggregates (data not shown).

The interaction of the DnaK system with small non-turbid aggregates was highly productive in terms of protein reactivation. This implies that unlike GroELS chaperonins, the DnaK system can bind locally and initiate assisted refolding in a local region of a polypeptide while still being involved in strong protein-protein interactions within the aggregate. Noticeably, protein reactivation by the DnaK system became increasingly futile as the complexity of the aggregate increased and the hydrophobic exposure decreased. This can be attributed to the reduced exposure of chaperone binding sites in large non-turbid aggregates and in turbid aggregates when compared with small soluble aggregates. Consistent with this interpretation is our finding that at the same concentration of aggregates (1 μM) only the fraction containing the lowest mass (not the medium

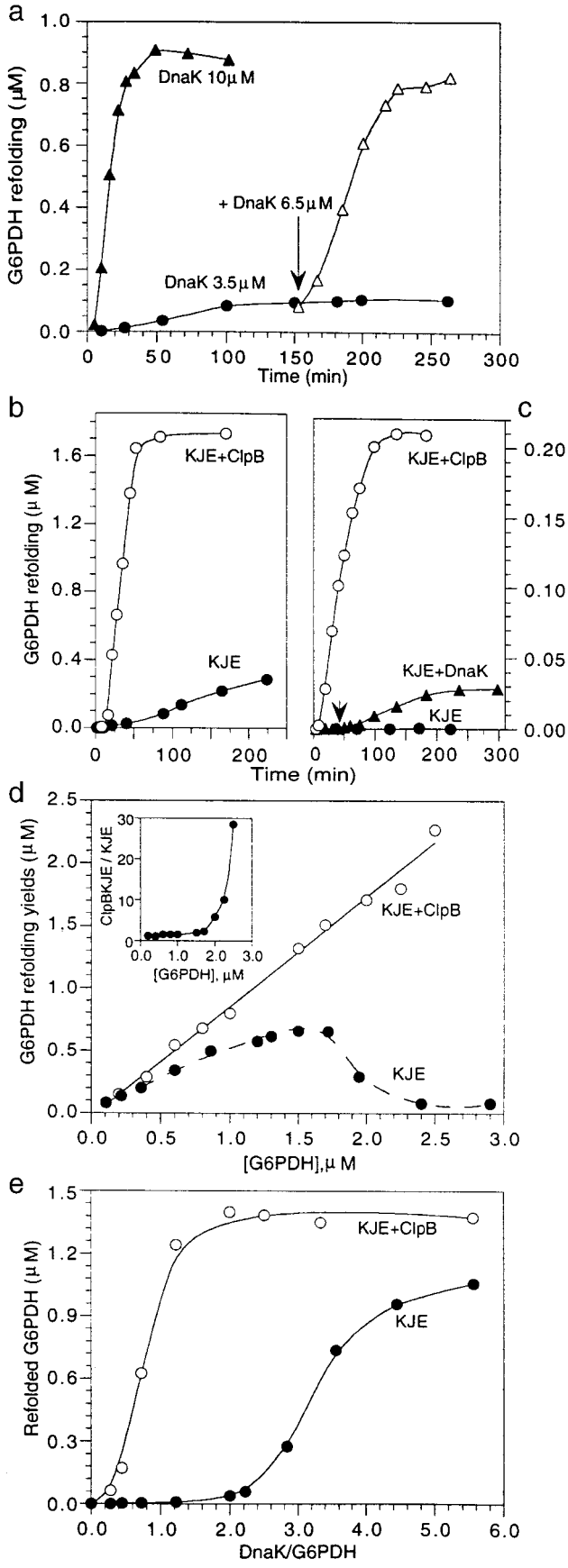

FIG. 5. **High DnaK or low ClpB concentrations activate DnaK-mediated refolding of large aggregates.** *a*, effect of DnaK concentrations. 2 μM non-turbid aggregates were reactivated in the presence of constant 0.7 μM DnaJ, 0.35 μM GrpE, and 3.5 μM DnaK (*filled circles*) or 10 μM DnaK added at time 0 (*filled triangles*) or 6.5 μM DnaK supplemented after 150 min of reaction (*arrow*) with low DnaK (*open triangles*). *b*, reactivation of 2 μM aggregates by DnaK, DnaJ, and GrpE (*KJE*) (3.5, 0.7, and 0.35 μM, respectively) without (*filled circles*) or with 0.5 μM ClpB (*open circles*). *c*, reactivation of 0.4 μM turbid aggregates (formed at 65 °C), by DnaK, DnaJ, and GrpE alone (*filled circles, KJE*) or supplemented after 40 min with 6.5 μM DnaK (*arrowhead, filled triangles*) or with 0.5 μM ClpB as in *a* (*open circles*). *d*, effect of concentration and size of G6PDH aggregates on the yields of chaperone-mediated reactivation by DnaK, DnaJ, and GrpE (3.5, 0.7, and 0.35 μM, respectively), without (*filled circles, KJE*) or with 0.5 μM ClpB (*open circles, KJE+C1pB*). *Inset,* activation effect of ClpB on DnaK-mediated disaggregation of increasingly large and abundant aggregates. *e*, dose-response curve of DnaK, DnaJ, and GrpE concentrations (constant molar ratio of 10:2:1 between co-chaperones) as a function of the yields of G6PDH reactivation from non-turbid large aggregates formed at 1.8 μM without (*filled circles*) or with 0.5 μM ClpB (*open circles*).

or large soluble aggregates isolated by gel filtration chromatography) was efficiently refolded by the DnaK system. On the other hand, although dilution did not affect the complexity of large aggregates, the yields of refolding were about 50 times higher with 0.2 μM than with 1 μM of the same large aggregates (Fig. 3, fractions A and B). Thus, a higher ratio of DnaK per G6PDH can compensate for the decreased efficiency of reactivation of large aggregates. Indeed, large aggregates were efficiently reactivated when the DnaK concentration was increased to a 5-fold excess (10 μM) over the substrate. A non-physiological increase of the concentration of only DnaJ or GrpE resulted in a strong inhibition of the chaperone activity with all types of aggregates (data not shown). However, as with DnaK alone, the inhibited reactivation was similarly alleviated by the concomitant increase in DnaK, DnaJ, and GrpE levels while keeping a fixed ratio of 10:2:1 between the respective members of the chaperone system (Fig. 5*e*).

Moreover, the dose response of the DnaK system indicates that in the absence of ClpB, the mechanism of productive disaggregation requires some sort of a cooperative behavior between different DnaK molecules because efficient refolding requires that more than one chaperone molecule interacts at the same time with the same polypeptide within the aggregate. Because the algorithm of Rüdiger *et al.* (21) predicts as many as 12 putative high affinity DnaK-binding sites, several DnaK molecules can theoretically bind to the same G6PDH polypeptide. Our data, however, suggest that as the aggregates became larger and more turbid, more sites seemingly became buried within the structure and were less available for DnaK binding.

A combination of the yeast chaperones hsp104, hsp70, and hsp40 initially demonstrated some disaggregation activity *in vitro* and solubilization of pre-aggregated proteins (9). The hsp104 homologue, ClpB, was further demonstrated to interact directly with large turbid protein aggregates, and together with ATP it induced structural changes in the aggregates. Hydrophobic regions became transiently exposed and available for subsequent interactions with the DnaK system leading to efficient disaggregation activity (12). We demonstrate in this study that for efficient solubilization of large aggregates the presence of ClpB reduces the need for an excess of DnaK. A dose response of the DnaK system in the presence of a constant concentration of substrate suggests that efficient reactivation with ClpB requires 4–5 times less DnaK per substrate than reactivation without ClpB.

There is no obvious Hsp100 homologue in the complete genome of *Drosophila melanogaster*. Our finding that protein disaggregation can take place with large amounts of Hsp70 even without Hsp100 correlates well with the exceptionally high levels of Hsp70 found in *Drosophila* under stress. Interestingly, Hsp70 overexpression suppresses polyglutamine aggregate-mediated neurodegeneration in *Drosophila* (31).

The Mechanism of DnaK-mediated Disaggregation—In the presence of ClpB, the mechanism of productive disaggregation exempts the DnaK molecules from having to act in an apparent cooperative manner (Fig. 5*e*). We suggest a two-step cooperative mechanism for the DnaK-DnaJ-GrpE-mediated solubilization and reactivation of stable protein aggregates. During the first step, several DnaK molecules must initially interact with exposed hydrophobic regions preferentially in the same aggregated polypeptide and thus prevent intramolecular collapses and intermolecular re-aggregation. During the second step, a dynamic binding and release of DnaK in the presence of DnaJ, GrpE, and ATP allows folding of previously bound regions, whereas the rest of the molecule is maintained in a partially non-collapsed state by other bound DnaK molecules. This would explain the observed apparent cooperative effect between DnaK molecules, which was canceled in the presence of ClpB. In Ref. 12, we proposed a mechanism in which ClpB hexamers shear ClpB-bound aggregates and thus actively expose some of the hidden hydrophobic regions on the aggregates. Hence, although through a different mechanism, ClpB could maintain aggregated polypeptides in a partially non-collapsed state and thus functionally surrogate the holding role otherwise carried out by excess DnaK. ClpB activity would thus free the excess DnaK molecules to instead perform repetitive cycles of productive assisted refolding (with DnaJ and GrpE) on local exposed regions in the aggregated polypeptide, leading to efficient disaggregation and renaturation.

Implications for the Mechanism of DnaK and for the Chaperone Network in the Cell—Together with the evidence that the DnaK system alone can bind and initiate the folding of polypeptides that are still aggregated, the efficient folding of large aggregates by near stoichiometric amounts of DnaK in the presence of ClpB strongly suggests that the mechanism of DnaK involves repetitive cycles of local rather than global assisted folding.

In the cell, the efficient rescue of thermolabile proteins during and after stress depends on the nature of the unfolded intermediates generated by each protein and on the availability and specificity of the various components of the chaperone network for unfolding intermediates. Thus, GroEL may preferentially interact with partially denatured molten globules (22), IbpB with extensively unfolded proteins (17), and ClpB with large turbid aggregates (12). In contrast, the DnaK system may interact with small segments of about 8–9 residues in extensively unfolded proteins (23) as well as in exposed polypeptide loops within large yet non-turbid protein aggregates (this work).

Because the DnaK system interacts with small extended segments, this chaperone system has been initially assigned to an early stage in the sequential folding pathway of nascent and newly translocated proteins prior to GroEL/GroES chaperonins (24). However, other studies have shown that the DnaK system can efficiently process folding intermediates handled from small heat shock proteins (17), components of the import complex of organelles (25, 26), and from GroEL (27). In addition, the DnaK system functionally cooperates with trigger factor (28), Hsp90 (29), and ClpB (12, 13). Our finding that small aggregates can be actively solubilized by the DnaK machinery has a major consequence for the role of the DnaK system within the chaperone network of the cell. It can (i) receive and process soluble intermediates from import pores, ribosomes, and other chaperones, (ii) resolubilize early forms of aggregates that accidentally form as by-products of the folding activity of other chaperones such as GroEL/GroES (30), and (iii) disaggregate small size aggregates generated during stress. Thus the hsp70 system can reintroduce polypeptides trapped in small aggregates back into the chaperone network for refolding or into the protease network for degradation.

Acknowledgments—We thank H-J. Schönfeld for purified DnaK, DnaJ, and GrpE, C. Squires for plasmid pClpB, and S. Rüdiger for predicting the number of DnaK-binding sites in G6PDH.

REFERENCES

1. Netzer, W. J., and Hartl, F. U. (1998) *Trends Biochem. Sci.* **23,** 68–73
2. Bukau, B., Schmid, F. X., and Buchner, J. (1999) in *Molecular Chaperones and Folding Catalysts. Regulation, Cellular Function and Mechanism* (Bukau, B., ed) pp. 3–10, Harwood Academic Publishers, Amsterdam
3. Bukau, B., and Horwich, A. L. (1998) *Cell* **92,** 351–366
4. Fink, A. L. (1999) *Physiol. Rev.* **79,** 425–449
5. Jaenicke, R., and Seckler, R. (1999) in *Molecular Chaperones and Folding Catalysts. Regulation, Cellular Function and Mechanism* (Bukau, B., ed) pp. 407–436, Harwood Academic Publishers, Amsterdam
6. Skowyra, D., Georgopoulos, C., and Zylicz, M. (1990) *Cell* **62,** 939–944
7. Ziemienowicz, A., Skowyra, D., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C., and Zylicz, M. (1993) *J. Biol. Chem.* **268,** 25425–25431
8. Schröder, H., Langer, T., Hartl, F.-U., and Bukau, B. (1993) *EMBO J.* **12,** 4137–4144
9. Glover, J. R., and Lindquist, S. (1998) *Cell* **94,** 73–82
10. Motohashi, K., Watanabe, Y., Yohda, M., and Yoshida, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7184–7189
11. Zolkiewski, M. (1999) *J. Biol. Chem.* **274,** 28083–28086
12. Goloubinoff, P., Mogk, A., Peres Ben-Zvi, A., Tomoyasu, T., and Bukau, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **23,** 13732–13737
13. Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., and Bukau, B. (1999) *EMBO J.* **18,** 6934–6949
14. Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Reinstein, J., and Bukau, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5452–5457
15. Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B., and Chung, C. H. (1992) *J. Biol. Chem.* **267,** 20429–20434
16. Török, Z., Horvath, I., Goloubinoff, P., Kovács, E., Glatz, A., Balogh, G., and László, V. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2192–2197
17. Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998) *J. Biol. Chem.* **273,** 11032–11037
18. Diamant, S., and Goloubinoff, P. (1998) *Biochemistry* **37,** 9688–9694
19. Mendoza, J. A., Warren, T., and Dulin, P. (1996) *Biochem. Biophys. Res. Commun.* **229,** 271–274
20. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature* **342,** 884–889
21. Rüdiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) *EMBO J.* **16,** 1501–1507
22. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) *Nature* **352,** 36–42
23. Rüdiger, S., Buchberger, A., and Bukau, B. (1997) *Nat. Struct. Biol.* **4,** 342–349
24. Hendrick, J. P., and Hartl, F. U. (1995) *FASEB J.* **9,** 1559–1569
25. Hohfeld, J., and Hartl, F.-U. (1994) *Curr. Opin. Cell Biol.* **4,** 499–509
26. Voisine, C., Craig, E. A., Zufall, N., von Ahsen, O., Pfanner, N., and Voos, W. (1999) *Cell* **97,** 565–574
27. Buchberger, A., Schröder, H., Hesterkamp, T., Schönfeld, H.-J., and Bukau, B. (1996) *J. Mol. Biol.* **261,** 328–333
28. Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999) *Nature* **400,** 693–696
29. Freeman, B. C., and Morimoto, R. I. (1996) *EMBO J.* **15,** 2969–2979
30. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1994) *Science* **265,** 659–666
31. Warrick, J. M., Chan, H. Y., Gray-Board, G. L., Chai, Y., Paulson, H. L., and Bonini, N. M. (1999) *Nat. Genet.* **23,** 425–428