The Importance of Having Thermosensor Control in the DnaK Chaperone System

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In addition to the σ22-mediated heat shock response, the DnaK/DnaJ/GrpE molecular chaperone system of Escherichia coli directly adapts to elevated temperatures by sequestering a higher fraction of substrate. This immediate heat shock response is due to the differential temperature dependence of the activity of DnaJ, which stimulates the hydrolysis of DnaK-bound ATP, and the activity of GrpE, which facilitates ADP/ATP exchange and converts DnaK from its high-affinity ADP-ligated state into its low-affinity ATP-ligated state. GrpE acts as thermosensor with its ADP/ATP exchange activity decreasing above 40 °C. To assess the importance of this reversible thermal adaptation for the chaperone action of the DnaK/DnaJ/GrpE system during heat shock, we used glucose-6-phosphate dehydrogenase and luciferase as substrates. We compared the performance of wild-type GrpE as a component of the chaperone system with that of GrpE R40C. In this mutant, the thermosensing helices are stabilized with an intersubunit disulfide bond and its nucleotide exchange activity thus increases continuously with increasing temperature. Wild-type GrpE with intact thermosensor proved superior to GrpE R40C with desensitized thermosensor. The chaperone system with wild-type GrpE yielded not only a higher fraction of refolding-competent protein at the end of a heat shock but also protected luciferase more efficiently against inactivation during heat shock. Consistent with their differential thermal behavior, the protective effects of wild-type GrpE and GrpE R40C diverged more and more with increasing temperature. Thus, the direct thermal adaptation of the DnaK chaperone system by thermosensing GrpE is essential for efficient chaperone action during heat shock.

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family participate in many cellular processes, including the folding, membrane translocation, and degradation of proteins. The chaperones recognize and interact with hydrophobic peptide segments, which are exposed by nascent polypeptide chains during synthesis and by misfolded proteins during stress, in particular heat shock. The irreversible formation of protein aggregates thus is reduced, increasing the yield of properly folded and refolded native protein. Hsp70 bind and release their substrates in an ATP-driven cycle (2, 3). The ATPase activity resides in the NH2-terminal domain (4, 5) and modulates the substrate binding properties of the COOH-terminal peptide-binding domain (6). The ATP-ligated T state is characterized by low affinity for substrates and fast rates of binding and release, whereas the ADP-ligated R state shows high affinity for substrates with slow kinetics (7, 8). DnaK, an Hsp70 homolog in Escherichia coli, acts in concert with its co-chaperones DnaJ, an Hsp40 homolog, and GrpE (2, 9, 10). DnaJ stimulates the hydrolysis of DnaK-bound ATP and converts T-state DnaK into its high-affinity R state (Fig. 1). GrpE facilitates ADP/ATP exchange and reconverts DnaK into the low-affinity T state. In the presence of ATP, the concerted action of the two co-chaperones DnaJ and GrpE controls the steady-state distribution of T-state and R-state DnaK and thus modulates the fraction of substrate sequestered by high-affinity R-state DnaK (9–11).

In a study on the temperature dependence of the structural and functional properties of the individual components of the DnaK chaperone system (12), GrpE has been found to undergo a reversible thermal transition in the physiologically relevant temperature range (transition midpoint at −48 °C) as observed with circular dichroism measurements and differential scanning calorimetry. In DnaK and DnaJ, no conformational changes have been observed between 15 and 48 °C. Consistent with the structural data, the rate of the DnaJ-triggered T→R conversion follows an Arrhenius temperature dependence, whereas the rate of the GrpE-dependent R→T conversion increases less and less with increasing temperature and even decreases above 40 °C (12). Similar studies of GrpE and its homolog from Thermus thermophilus have confirmed the occurrence of a reversible thermal transition (13, 14). Stabilization of the pair of the long NH2-terminal helices in the GrpE dimer with an engineered disulfide bond (R40C) (Fig. 2) abolishes the thermal transition in GrpE and reduces the deviation of the ADP/ATP exchange activity from an Arrhenius temperature dependence, indicating that the long helix pair acts as the primary thermosensor of the chaperone system (15, 16).

In E. coli, DnaK and its co-chaperones are constitutively expressed as well as induced by heat shock or other cellular stress. This familiar heat shock response is mediated by σ22, which directs RNA polymerase to transcribe the particular set of heat shock genes. Within minutes after heat shock, the cellular concentrations of DnaK, DnaJ, and GrpE are increased 2-fold (17, 18). Recent experiments with the isolated DnaK/DnaJ/GrpE/ATP chaperone system have shown that a mere increase in temperature suffices to sequester a higher fraction of fluorescence-labeled peptide substrates (11). This direct thermal response of the chaperone system is due to the differential temperature dependence of the two
Thermosensor Control of DnaK Chaperone System

EXPERIMENTAL PROCEDURES

Materials—DnaK was expressed and purified as described previously (19). Its concentration was determined photometrically by measuring the increase in NADH concentration at 340 nm for 30 s with an HP 8453 spectrophotometer at 25 °C. The activity of G6PDH was determined photometrically by measuring the increase in NADH concentration at 340 nm for 30 s with an HP 8453 spectrophotometer at 25 °C. The assay volume was 1 ml and contained 3 mM glucose 6-phosphate and 0.15 mM NADH in 200 mM Hepes/NaOH, pH 7.6. The reaction was started by adding 15 μl of the G6PDH-containing sample solution. Luciferase activity was measured in a Lumac Biocounter M1500 as described previously (23).

RESULTS AND DISCUSSION

The DnaK Chaperone System Prevents Protein Aggregation during Heat Shock—Upon heat shock, labile proteins are denatured and either are irreversibly trapped in aggregates or remain in a refolding-competent state. The formation of protein aggregates upon heat denaturation has been measured in vitro by several methods including sedimentation analysis, light scattering, and Congo Red or thioflavin T staining (24–27). The fraction of aggregated protein that is detected depends on the analytical method used as well as the solubility, size, and shape of the aggregates formed. In this study, we used enzymes as substrates and determined the fraction of refolding-competent protein rather than that of aggregated protein, i.e. we measured the amount of active enzyme recovered after heat shock.

To assess the effect of the individual components of the DnaK chaperone system on preventing protein aggregation during heat shock, we subjected the protein substrates to heat shock in the presence of different combinations of chaperones and then let it refold with the complete DnaK/DnaJ/GrpE/ATP chaperone system present. Any observed differences in the yield of refolding thus could be unequivocally attributed to the differential conditions during heat shock. As protein substrates, we used G6PDH and luciferase. Both enzymes had previously been shown to form stable protein aggregates at heat shock temperatures as observed with size exclusion chromatography and light scattering (25, 28). G6PDH, which is heat-stable, was

FIG. 1. The DnaK/DnaJ/GrpE chaperone cycle. The substrate is fed into the cycle by fast binding to ATP-ligated T-state DnaK. Direct binding to ADP-ligated R-state DnaK is too slow to be of physiological significance (2, 8). DnaJ-stimulated hydrolysis of DnaK-bound ATP locks DnaK onto the substrate (30, 31). Direct release of substrate from ATP-ligated R-state DnaK is negligibly slow (8). GrpE-catalyzed ADP/ATP exchange completes the cycle and releases the substrate. Under heat shock conditions, the substrate is dynamically sequestered with high-affinity DnaK due to a shift of the co-chaperone-controlled steady state of the system toward the R state (11, 15).

FIG. 2. The nucleotide exchange factor GrpE. The GrpE G122D dimer has been co-crystallized with the ATPase domain of DnaK(5). To date, the crystal structure of WT GrpE itself is not available. α-Helices and β-sheets in GrpE are represented by ribbons, and residues not resolved in the crystal structure are denoted with dashed lines. The ATPase domain of DnaK is indicated with a shaded contour. The solid bar indicates the position of the newly introduced disulfide bond in GrpE R40C. C, COOH terminus; N, NH2 terminus. The atomic coordinates are from Protein Data Bank entry 1DKG.

co-chaperones, resulting in a shift toward R-state DnaK with high affinity for substrates. Here, we compare the co-chaperone efficiencies of wild-type (WT) GrpE with intact thermosensor and GrpE R40C with desensitized thermosensor during heat shock. The DnaK system with WT GrpE proved superior to that with GrpE R40C in protecting protein substrate against both heat inactivation and aggregation, indicating the importance of the GrpE thermosensor for efficient chaperone action under heat shock conditions.
The refolding-competent G6PDH was chaperones had been present during heat shock, the fraction of absence of either DnaJ or GrpE during heat shock. When no already been present during heat shock. If ATP had been turation if the complete chaperone system including ATP had during the heat shock, G6PDH was exposed to a temperature of 45 °C for 15 min in the presence of various combinations of the compo- nents of the chaperone system. During the heat shock, G6PDH was reactivated at 25 °C in the presence of the fully active chaperone system including ATP and varying GrpE concentra- tions. Subsequently, apyrase was added and the sponta- neous refolding of G6PDH at 25 °C was monitored. The fraction of refolding-competent G6PDH depended on the concentration of GrpE (Fig. 4A). The maximum yield of reactivated G6PDH was obtained at a GrpE concentration of ~1 μM (Fig. 4B). Both lower and higher GrpE concentrations resulted in a lower amount of refolded G6PDH. GrpE shifts the steady state of the DnaK ATPase cycle from the R state toward the T state. Apparently, maximum protection of G6PDH against aggregation requires an optimum steady-state R-state/T-state ratio. The thermosensor control of GrpE thus may be assumed to have an effect on the prevention of protein aggregation during heat shock provided that a temperature-induced decrease in nucleotide exchange activity of GrpE (see Fig. 4E) has the same effect as a decrease in its concentration.

In the following series of experiments, we examined with a similar set-up the role of the thermosensor action of GrpE in the protection of protein substrate against aggregation during heat shock. We compared the co-chaperone efficiency of WT GrpE with that of GrpE R40C (Fig. 2) in which the pair of the thermosensing helices had been stabilized with an intersub- unit disulfide bond at their NH2 termini (15). In GrpE R40C, no thermal transition is observed in the physiological temperature range. The temperature dependence of the ADP/ATP exchange activity of WT and disulfide-stabilized GrpE is similar between 15 and 40 °C. Above this temperature range, the stabilizing effect of the interhelical cross-link in GrpE R40C becomes manifest and the rate of the R→T conversion increases continuously (15), resulting in an increasing difference in nucleotide exchange activity between WT and mutant GrpE with increasing temperature (Fig. 4E). To normalize their co-chaperone activities at low temperature, we monitored the refolding of heat-treated G6PDH at 25 °C as a function of the concentration of WT GrpE and GrpE R40C in the presence of DnaK, DnaJ, and ATP. The yield of enzymic activity was found to be GrpE-dependent (Fig. 4C), consistent with previous studies (27, 29, 32). If the concentration of GrpE R40C was divided by 1.2, the reactivation pattern of G6PDH in the presence of GrpE R40C coincided with that in the presence of WT GrpE (Fig. 4D). Apparently, at 25 °C, GrpE R40C possesses a 1.2 times lower

In analogous experiments, thermolabile luciferase (25) was directly inactivated by heat denaturation at 45 °C (Fig. 3B). After heat shock, luciferase was reactivated at 25 °C to ~75% of the initial activity if the complete chaperone system includ- ing ATP had been present during heat shock. Almost no refolding occurred if DnaJ or GrpE during heat shock. When no chaperones had been present during heat shock, the fraction of refolding-competent luciferase decreased to ~50%. Apparently, the prevention of aggregation of both G6PDH and luciferase is a dynamic process requiring a continuously running T-R cycle of DnaK. The control of the ATP-driven chaperone cycle not

**FIG. 3.** The DnaK chaperone system prevents protein aggregation during heat shock. A, chemically denatured G6PDH (see “Experimental Procedures”) was incubated at 45 °C for 15 min in the presence of the indicated components of the chaperone system: DnaK, DnaJ, GrpE, and ATP (●); DnaK, DnaJ, and ATP (▲); DnaK, GrpE, and ATP (▼); and no chaperone and no ATP (▲). Final concentrations (as indicated throughout) were 250 nM G6PDH, 3.5 μM DnaK, 0.7 μM DnaJ, 0.35 μM GrpE, and 10 mM ATP. After heat shock, the samples were complemented with the lacking components of the DnaK/DnaJ/GrpE/ATP chaperone system and incubated at 25 °C. The enzymic activity of G6PDH was measured during and after heat shock at different time points and expressed as percentage of the initial activity. The data points were fitted with a double-exponential equation. B, an analogous experiment was performed with 250 nM luciferase. In the case of heat-labile luciferase, the chemical denaturation preceding the heat shock was omitted.

denatured in 6 M guanidine hydrochloride. After dilution of the denaturant, G6PDH was exposed to a temperature of 45 °C for 15 min in the presence of various combinations of the components of the chaperone system. During the heat shock, G6PDH remained inactive (Fig. 3A) (see also Ref. 29). After heat shock, G6PDH was reactivated at 25 °C in the presence of the fully active chaperone system to ~60% of the activity prior to dena- turation if the complete chaperone system including ATP had already been present during heat shock. If ATP had been absent during heat shock, the yield of active enzyme was only ~30% of the initial activity. The same yield was obtained in the absence of either DnaJ or GrpE during heat shock. When no chaperones had been present during heat shock, the fraction of refolding-competent G6PDH was <10%.

In analogous experiments, thermolabile luciferase (25) was directly inactivated by heat denaturation at 45 °C (Fig. 3B). After heat shock, luciferase was reactivated at 25 °C to ~75% of the initial activity if the complete chaperone system includ- ing ATP had been present during heat shock. Almost no refolding occurred if DnaJ or GrpE during heat shock. In the absence of GrpE during heat shock, the fraction of refolding-competent luciferase decreased to ~50%. Apparently, the prevention of aggregation of both G6PDH and luciferase is a dynamic process requiring a continuously running T-R cycle of DnaK. The control of the ATP-driven chaperone cycle not only by DnaJ (30, 31) but also by GrpE (9, 10) is essential for maximum protection of substrate protein against aggregation.

**Thermosensor Effect of GrpE in the Prevention of Protein Aggregation during Heat Shock**—Under heat shock conditions, the differential temperature dependence of the activities of the co-chaperones DnaJ and GrpE becomes manifest. The rate of the DnaJ-stimulated T→R conversion increases exponentially with increasing temperature, whereas the rate of the GrpE- facilitated R→T conversion increases less and less and even decreases above 40 °C (Fig. 4E) (12). The resulting shift of DnaK toward its R state increases the fraction of substrate that is sequestered by high-affinity DnaK (11). In a first series of experiments, we investigated the protective effect of the chaperone system against protein aggregation as a function of GrpE concentration. To focus on the heat shock phase and to minimize the chaperone effect on the protein substrates during the refolding phase, we inhibited the chaperone system after the heat shock by depleting it of ATP and measured the amount of spontaneously refolding protein sub- strate (instead of providing identical refolding conditions after heat shock as in Fig. 3). For removing ATP, we used apyrase, an ATP diphosphohydrolase that hydrolyzes ATP into AMP and inorganic phosphate. Apyrase completely inhibits the chaperone-assisted refolding (Fig. 4F). Chemically denatured G6PDH was subjected to a temperature of 48 °C for 15 min in the presence of DnaK, DnaJ, ATP, and varying GrpE concentra- tions. Apparently, at 25 °C, GrpE R40C possesses a 1.2 times lower
co-chaperone activity than WT GrpE, consistent with its previously observed somewhat lower nucleotide exchange activity (15). To examine whether desensitization of the thermosensor in GrpE affected the prevention of protein aggregation during heat shock, we performed the experiment of Fig. 4B with GrpE R40C instead of WT GrpE. At GrpE concentrations >1 μM, the yield of refolding-competent G6PDH was lower in the presence of GrpE R40C than in the presence of WT GrpE, the WT
Efficiency of the Thermosensor Control by GrpE as a Function of Temperature—The rate of the WT GrpE-catalyzed R→T conversion of DnaK progressively decreases above 40 °C with increasing temperature, whereas in the presence of GrpE R40C, the rate continues to increase (see Fig. 4E). The ADP/ATP exchange activities of WT and disulfide-stabilized GrpE thus diverge more and more with increasing temperature above 40 °C. To examine whether the difference between WT GrpE and GrpE R40C in preventing protein aggregation during heat shock as observed in the experiments of Figs. 4B and 5B is indeed due to their divergent nucleotide exchange activities at higher temperature, we studied their efficiency as co-chaperone in the prevention of protein aggregation as a function of temperature. G6PDH was exposed to temperatures from 42 to 49 °C for 15 min in the presence of DnaK, DnaJ, ATP plus WT GrpE or GrpE R40C. The spontaneous refolding of G6PDH at 25 °C then was measured after the addition of apyrase. After a heat shock at 42 °C, the yield of G6PDH activity in the case of WT GrpE (activityWT) was about the same as that in the presence of GrpE R40C (activityR40C), resulting in an activityWT/activityR40C ratio of ~1 (Fig. 6A). With increasing temperature, however, the advantage of WT GrpE over GrpE R40C became increasingly evident. When luciferase was used as substrate, similar results were obtained (Fig. 6B). The increase in the activityWT/activityR40C ratio with increasing temperature appears to be linked with the differential thermal behavior of WT GrpE and GrpE R40C (Fig. 4E). Thus, the superior performance of WT GrpE as compared with GrpE R40C (Figs. 4 and 5) is to be ascribed to the intact thermosensor control of the chaperone system.

Effect of GrpE Concentration on the Retardation of Luciferase Heat Inactivation—Is the immediate thermal adaptation of the chaperone system, i.e. the effect of the GrpE thermosensor control, also observable directly during heat shock? We followed the loss of activity of luciferase at 44 °C as a function of GrpE concentration in the presence of DnaK, DnaJ, and ATP. The rate of heat inactivation of luciferase was found to depend on GrpE concentration (Fig. 7A). The maximum protective effect of the chaperone system was found again at a GrpE concentration of 1 μM (Fig. 7B). Thus, the dose-response curve
of GrpE in this experiment was similar to that obtained by measuring the amount of refolded luciferase after heat shock (see Fig. 5B). Apparently, GrpE-dependent T→R shifting of the steady state of the DnaK ATPase cycle not only affects the fraction of refolding-competent protein after heat shock but also the rate of heat inactivation of protein substrate.

Thermosensor Effect of GrpE in the Retardation of Heat Inactivation of Luciferase—To explore whether WT GrpE with intact thermosensor retarded the heat inactivation of protein substrate to a greater extent than GrpE R40C, we subjected luciferase to a temperature of 48 °C in the presence of DnaK, DnaJ, ATP plus WT GrpE or GrpE R40C and monitored the loss of luciferase activity. The chosen concentrations of WT and mutant GrpE were in the range either below or above 1/18528M, which is the optimum concentration for protecting luciferase against heat inactivation (see Fig. 7B). After heat exposure at low GrpE concentrations, the activity of luciferase in the case of WT GrpE (activityWT) was lower than that in the case of GrpE R40C (activityR40C), the activityWT/activityR40C ratio being 0.8 (Table I). At high GrpE concentrations, WT GrpE retarded the inactivation of luciferase during heat shock to a greater extent than GrpE R40C, the activityWT/activityR40C ratio being 1.4. Thus, the beneficial effect of the thermosensor control of the chaperone system during heat shock is not only evident from the yield of refolding-competent protein after heat shock but also from the retardation of protein inactivation during heat shock.

CONCLUSION

The ATPase cycle of DnaK is controlled by the two co-chaperones DnaJ and GrpE, which through stimulation of ATP hydrolysis and ADP/ATP exchange promote substrate binding and release, respectively. The DnaK/DnaJ/GrpE/ATP machinery exerts two apparently dissimilar chaperone functions, i.e. the refolding of denatured proteins and the prevention of protein aggregation. Both processes depend on a continuously running chaperone cycle and seem not to be always equally important. The folding or refolding of proteins under normal growth conditions requires an optimally tuned binding and release cycle, which is primarily controlled by DnaJ. The formation of ternary (ATP/DnaK)DnaJ complexes increases the rate of ATP hydrolysis by one to two orders of magnitude, locking ADP/DnaK onto the substrate polypeptide (30, 31). Thus, DnaJ monitors the concentration of non-native proteins and feeds them into the chaperone cycle (31). At heat shock temperatures, however, prevention of protein aggregation through enhanced sequestering of misfolded polypeptide segments by DnaK seems to become more important. Heat-sensing GrpE (12–14) joins DnaJ in controlling the chaperone cycle. Reduction of its nucleotide exchange activity results in a T→R shift of DnaK and thus in a higher fraction of substrate being sequestered by high-affinity DnaK (11). This sequestering is also a dynamic process exerted through a rapid T-R cycle of the chaperone system. In this study, we show (i) that an optimum GrpE concentration exists for the assistance of folding/refolding as well as the prevention of heat aggregation of proteins and (ii) that the thermosensor control of the DnaK/DnaJ/GrpE system indeed improves its chaperone effect at heat shock conditions. Desensitization of the thermosensor by
Thermosensor effect of GrpE in the retardation of heat inactivation of luciferase

Luciferase (250 nm) was incubated at 48 °C in the presence of 3.5 μM DnaK, 0.7 μM DnaJ, 10 mM ATP, plus WT GrpE or GrpE R40C. The loss in enzymic activity was measured as a function of time. Luciferase activity at 7.5 min in the presence of WT GrpE (activityWT) or GrpE R40C (activityR40C) was obtained by interpolating the data points with a double-exponential equation (compare with data in Fig. 7). The GrpE concentrations were <1 μM (0.5 μM WT GrpE or 0.6 μM GrpE R40C) and >1 μM (4.0 μM WT GrpE or 4.8 μM GrpE R40C), the GrpE R40C concentrations being 1.2 times higher than those of WT GrpE (for explanation, see legend to Fig. 5D). The means ± S.D. from four experiments are shown. The value of the ratio corresponds to the average of the ratios from the four data pairs.

| GrpE concentration | ActivityWT | % initial | ActivityR40C | ActivityWT/activityR40C ratio |
|-------------------|------------|----------|--------------|-----------------------------|
| Low (<1 μM)       | 0.106 ± 0.010 | 100     | 0.133 ± 0.006 | 0.81 ± 0.07                |
| High (>1 μM)      | 0.106 ± 0.010 | 100     | 0.133 ± 0.006 | 1.43 ± 0.04                |

stabilizing the long helix pair in the GrpE dimer with an N-domain terminal intersubunit disulfide bond (GrpE R40C) (Fig. 2) impaired the protection of protein substrates during heat shock.

Importantly, the advantageous effect of the thermosensor control by GrpE may be assumed to be actually much larger than is manifest in the experiments with GrpE R40C (Figs. 4B, 5B, and 6), because the cross-linked helices in GrpE R40C do not convert GrpE into a heat-stable protein with an activity that exponentially increases with temperature (see Fig. 4E). Such an Arrhenius temperature dependence is, for instance, observed for the spontaneous nucleotide exchange in DnaK or for the DnaJ-stimulated hydrolysis of DnaK-bound ATP (12). Thus, GrpE R40C is an imperfect surrogate for a nucleotide exchange factor with normal Arrhenius temperature dependence.

GrpE, in contrast to DnaJ (35, 36), does not act as a chaperon on its own. Nevertheless, it is the only member of the DnaK/DnaJ/GrpE system that is essential for bacterial growth at all temperatures (37), emphasizing the importance of thermosensor control of the ATPase cycle in vivo. In a recent study (38) with a GrpE-deficient strain, the recombination of recombinant GrpE has been varied by changing the concentration of the inducer. Too little as well as too much GrpE reduced cellular growth, underlining again the importance of an optimally controlled ATPase cycle of DnaK. The DnaK system in E. coli and its homolog in T. thermophila (13) are not the only chaperone systems that take advantage of direct thermal control. Most recently, the en-oplasmic reticulum-resident chaperone calreticulin was shown to undergo a thermal transition, which enhances its ability to bind polypeptide substrates and improves the prevention of protein aggregation during heat shock (39). The occurrence in all three biological kingdoms emphasizes the importance of direct thermal control in chaperone systems.

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