Reversible Thermal Transition in GrpE, the Nucleotide Exchange Factor of the DnaK Heat-Shock System*

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DnaK, a Hsp70 acting in concert with its co-chaperones DnaJ and GrpE, is essential for Escherichia coli to survive environmental stress, including exposure to elevated temperatures. Here we explored the influence of temperature on the structure of the individual components and the functional properties of the chaperone system. GrpE undergoes extensive but fully reversible conformational changes in the physiologically relevant temperature range (transition midpoint at ~48 °C), as observed with both circular dichroism measurements and differential scanning calorimetry, whereas no thermal transitions occur in DnaK and DnaJ between 15 °C and 48 °C. The conformational changes in GrpE appear to be important in controlling the interconversion of T-state DnaK (ATP-ligated, low affinity for polypeptide substrates) and R-state DnaK (ADP-ligated, high affinity for polypeptide substrates). The rate of the T → R conversion of DnaK due to DnaJ-triggered ATP hydrolysis follows an Arrhenius temperature dependence. In contrast, the rate of the R → T conversion due to GrpE-catalyzed ADP/ATP exchange increases progressively less with increasing temperature and even decreases at temperatures above ~40 °C, indicating a temperature-dependent reversible inactivation of GrpE.

At heat-shock temperatures, the reversible structural changes of GrpE thus shift DnaK toward its high-affinity R state.

Chaperone systems of the Hsp70 family facilitate the folding of nascent polypeptide chains and denatured proteins, preventing the formation of protein aggregates (for a comprehensive review, see Ref. 1). DnaK, a Hsp70 homolog of Escherichia coli, binds peptides and segments of denatured proteins in extended conformation (2, 3). In its chaperone action, DnaK cooperates with two cohort heat-shock proteins, DnaJ and GrpE (4). The DnaK chaperone system has been studied extensively in vitro at ambient temperatures. The model cycle of the system may be summarized as follows (Fig. 1; Refs. 5–11): DnaK alternates between two states, the ATP-ligated T state and the ADP-ligated R state. The conversion of DnaK from its T state to the R state is mediated by DnaJ, which facilitates the hydrolysis of DnaK-bound ATP. The conversion from the R state back to the T state is triggered by GrpE, which facilitates the exchange of DnaK-bound ADP for ATP. The affinity of T-state DnaK for peptide and protein substrates is low, and both binding and release of substrates are fast. In contrast, the substrate affinity of R-state DnaK is high, and the rates of binding and release of substrates are too slow to be of physiological significance. Thus, a substrate is first bound by T-state DnaK, which is then converted to the high-affinity R state in a DnaJ-triggered reaction. With the assistance of GrpE, DnaK is re-converted into the low-affinity T state, releasing the substrate.

The expression of DnaK and the co-chaperones DnaJ and GrpE is controlled by the transcription factor σ32 (for a review, see Ref. 12). The expression levels of chaperones and co-chaperones are enhanced by heat shock, resulting in an approximately 2-fold increase in the cellular concentrations of DnaK (13) and the co-chaperones. 1 Here we report a study of the effect of heat-shock conditions on the DnaK chaperone system itself. We found the co-chaperone GrpE to undergo a reversible conformational transition within the physiologically relevant temperature range that appears to be of functional significance.

EXPERIMENTAL PROCEDURES

Materiales—DnaK was purified as described previously (6) and stored at ~80 °C. To prepare stock solutions for experimentation, samples containing ~12 mg of DnaK were thawed, concentrated by ultrafiltration (Centricon-30; Amicon) to 500 μl, and transferred to assay buffer (25 mM Hepes/NaOH, 100 mM KCl, 10 mM MgCl2, pH 7.0, or 25 mM potassium phosphate, pH 7.0, for circular dichroism experiments) by size exclusion chromatography (NAP-10; Amersham Pharmacia Biotech). The protein concentration was determined photometrically with a molar absorption coefficient of ε280 = 14,500 M⁻¹ cm⁻¹ (14). The concentrations of DnaK stock solutions were ~100 μM. The nucleotide content was <0.1 μmole nucleotide/mol DnaK (15). DnaJ (110 μM; in 50 mM Tris-HCl, pH 7.7) and GrpE (200 μM; in 50 mM Tris-HCl, pH 7.7) were prepared as described elsewhere (16, 17). For circular dichroism measurements and differential scanning calorimetry, GrpE was dialyzed against 25 mM potassium phosphate, pH 7.0.

The amino acid sequence of peptide a-la-p5 (ALLLSAPRR) is derived from the 23-residue prepiece of mitochondrial aspartate aminotransferase of chicken, which has previously proven to be a high-affinity ligand for DnaK (6, 18). Peptide a-la-p5 is labeled with acrylodan (6-acryloyl-2-dimethylaminonaphtalene) at the N-amino group. Synthesis and labeling of the peptide (purity >95%) are described elsewhere (14). The concentrations of the stock solutions of a-la-p5 and a-la-p5 were 1.9 mM in water and 240 μM in 20% (v/v) acetonitrile, respectively. N9-(4-N'-methylanthraniloylaminobutyl)-8-aminoadenosine 5’-diphosphate (MABA-ADP) was a gift from Dr. J. Reinstein (Max Planck Institut für molekulare Physiologie, Dortmund, Germany) and had been synthesized as described elsewhere (9). A fresh ATP stock solution (50 mM disodium salt, pH 7.0; Fluka) in assay buffer was prepared before every experimental series. The ADP stock solution was 38 mM ADP (disodium salt; Fluka), 50 mM potassium phosphate, pH 7.0.

Circular Dichroism Measurements—Circular dichroism was measured by guest on July 24, 2018http://www.jbc.org/Downloaded from

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1 X. Liu, P. Christen, H.-J. Schönfeld, and E. Sandmeier, manuscript in preparation.
2 The abbreviation used is: MABA-ADP, N9-(4-N'-methylanthraniloylaminobutyl)-8-aminoadenosine 5’-diphosphate.
Reversible Thermal Transition in GrpE

**RESULTS**

**Temperature-induced Structural Transitions in DnaK, DnaJ, and GrpE**—The thermal transitions in the secondary structure of DnaK and its co-chaperones were monitored with circular dichroism spectroscopy. GrpE was further examined by differential scanning calorimetry. In ADP-ligated DnaK, no structural changes were observed between 15 °C and 48 °C. At temperatures above the physiologically relevant range, two thermal transitions take place (Fig. 2): the midpoint of the low-temperature transition is at 58 °C, and the midpoint of the high-temperature transition is at 75 °C. In the first thermal transition, ~40% of the total ellipticity at 222 nm is lost; the residual secondary structure is abolished in the high-temperature transition. The structural transitions become partially irreversible when DnaK is heated to 95 °C. The stability of nucleotide-free DnaK is, as observed previously (20, 21), significantly lower than that of the nucleotide-ligated form; in the absence of nucleotide the first transition midpoint is shifted toward lower temperature by 9 °C (data not shown). Similar to nucleotide-ligated DnaK, DnaJ undergoes no structural changes between 15 °C and 48 °C (Fig. 3). A single thermal transition with a midpoint at ~58 °C is observed. The denaturation is irreversible when DnaJ is heated to 95 °C.

GrpE undergoes two well-separated temperature-induced conformational transitions, with midpoints at ~48 °C and 75–80 °C, as evident from both circular dichroism measurements and differential scanning calorimetry (Figs. 4 and 5). Experiments at varying concentrations (5–75 μM) of GrpE, which exists as a dimer (Fig. 1B; Refs. 16 and 22), indicated the low-temperature transition midpoint to be concentration-independent, consistent with a monomolecular unfolding process (data not shown). The unfolding, as monitored by both loss of ellipticity and change in molar heat capacity, was completely reversible after heating up to 60 °C (Fig. 5). The low-temperature heat capacity peak was broad and asymmetric, indicating that either part of the protein unfolds in a noncooperative manner or several tightly coupled cooperative transitions occur. In the low-temperature transition, ~25% of the total heat is absorbed. Unlike DnaK and DnaJ, GrpE loses a significant fraction of ellipticity below 48 °C (~40% of total). A rigorous thermodynamic analysis of the complete unfolding mechanism of GrpE is beyond the scope of the present study and will be the subject of further investigation.

**Temperature Dependence of the Rates of T/R Interconversion**—We examined the effects of temperature within the physiologically relevant range on the co-chaperone-induced interconversion of DnaK between its low-affinity T state and its high-affinity R state. The rates of these conversions were determined at fixed temperatures during a stepwise increase in temperature from 15 °C to 48 °C and then at fixed temperatures during stepwise cooling of the same solution. Three different types of measurements were performed: (i) DnaK possesses a single tryptophan residue at position 102 that allows spectroscopic monitoring of conformational changes (5, 10, 21); (ii) the increase in fluorescence intensity of acrylodan-labeled peptide a-ala-p5 upon binding of the peptide to DnaK (6) affords another possibility for monitoring the T/R interconversion of DnaK, which is accompanied by changes in the affinity for target peptides (Fig. 1A; Ref. 23); and (iii) the release of fluorescence-labeled ADP allows the monitoring of the rate-determining step of the nucleotide exchange, which takes place during the R → T conversion (9).

When the DnaJ-triggered T → R conversion was followed by the increase in the intrinsic fluorescence of DnaK, the rates of the conversion as a function of temperature complied with the Arrhenius equation (Fig. 6A). The conversion reactions were performed at 0.5 and 1 μM DnaJ; at all temperatures, their rates proved to be a linear function of the concentration of DnaJ. If we followed the increase in acrylodan fluorescence of peptide a-ala-p5 during the T → R conversion (Fig. 6B), we observed the same pattern of temperature dependence seen when the intrinsic fluorescence was monitored, although the observed rate constants were somewhat slower.

In contrast, if the temperature dependence of the rate of the GrpE-catalyzed R → T conversion was examined, a reversible temperature-dependent inactivation of GrpE became apparent that above ~40 °C resulted in decreasing reaction rates (Fig. 7A). The reactions, followed by the decrease in the intrinsic fluorescence of DnaK, were performed at different concentra-
tions of GrpE; at all temperatures, their rates were a linear function of the concentration of GrpE. At all concentrations of GrpE, the fastest rates were measured at 40 °C. Similar reaction rates and the same non-Arrhenius temperature dependence were observed when the decrease in acrylodan fluorescence of peptide a-ala-p5 was followed during the GrpE-catalyzed R→T conversion (Fig. 7B). The non-Arrhenius temperature dependence was also observed when the release of fluorescent MABA-ADP was followed (Fig. 7C).

The conformation and peptide binding properties of nucleotide-free DnaK are comparable with those of ADP-ligated, i.e. R-state, DnaK (9, 10). The R→T conversion of DnaK can thus be triggered by the addition of ATP to nucleotide-free DnaK rather than by the addition of GrpE. This experimental setup allows the examination of whether the non-Arrhenius temperature dependence observed in the R→T conversion (Fig. 7) is inherent to the R→T conversion or has to be attributed to the participation of GrpE in this process. The rates of the R→T transition triggered by the addition of ATP to nucleotide-free DnaK in the absence of GrpE and monitored by the decrease in the intrinsic fluorescence of DnaK exhibited an Arrhenius temperature dependence (Fig. 8A). The rates were more than 1 order of magnitude faster than those of the R→T conversion induced by the GrpE-catalyzed exchange of nucleotide (Fig. 7A), in which, under the chosen conditions, the GrpE-catalyzed release of nucleotide is rate-limiting. When the R→T conver-
Our experiments addressed the question of how temperature influences the structure of the components of the DnaK chaperone system (DnaK, DnaJ, and GrpE) and the kinetics of the co-chaperone-controlled T/R interconversion of DnaK. The experiments were performed over a temperature range from 15 °C up to the extreme heat-shock temperature of 48 °C, which approximately corresponds to the upper temperature boundary for growth of *E. coli* (24).

Nucleotide-ligated DnaK proved to be stable against thermal denaturation within this temperature range. In accordance with the stability of nucleotide-ligated DnaK, the steady-state ATPase activity of DnaK in the absence of co-chaperones shows Arrhenius behavior within the physiologically relevant temperature range (25). At higher temperatures, ADP-ligated DnaK shows two structural transitions (Fig. 2). The first transition has been assigned to the NH$_2$-terminal ATPase domain because the isolated ATPase domain undergoes a similar temperature a thermal transition that is absent in the isolated peptide-binding domain (20), a notion that has not remained undisputed (28). The co-chaperone DnaJ was also found to be stable up to 48 °C (Fig. 3), a finding that is consistent with previous studies (27). The very minor loss in ellipticity that was observed during heating and cooling of DnaK and DnaJ between 15 °C and 48 °C might be due to the fact that the midpoint of the first thermal transition, as determined by differential scanning calorimetry, is at 54.4 °C and
The reaction was started by mixing the following solutions: [1 mM DnaK, 4 mM ADP/Pi, and 2 mM a-ala-p5] + [GrpE and 5 mM ATP]. The concentrations of GrpE were 1 μM (○, △), 500 nM (▲, □), or 100 nM (■, □). All concentrations indicated are those after mixing. The first set of data points (○, △, ■) was obtained during a stepwise increase in temperature from 15 °C to 48 °C; the second set of data points (▲, □, □) was obtained during the decrease in temperature of the same solution. Solid lines indicate the Arrhenius curves calculated on the basis of the rates at 15 °C and 25 °C. Dotted lines have been added as a guide to the eye. For details, see “Experimental Procedures.” Inset, Arrhenius plots. B, the rates of the R → T conversion were determined by monitoring the decrease in acrylodan fluorescence of peptide a-ala-p5 at the indicated temperatures. The reaction was started by mixing the following solutions: [1 mM DnaK, 4 μM ADP/Pi, and 100 nM a-ala-p5] + [1 μM GrpE and 5 mM ATP]. C, the rates of the R → T conversion were determined by monitoring the decrease in MABA-ADP fluorescence at the indicated temperatures. The reaction was started by mixing the following solutions: [1 μM DnaK, 0.5 μM MABA-ADP, 3.5 μM ADP/Pi, and 2 μM a-ala-p5] + [1 μM GrpE and 5 mM ATP].

53 °C for DnaK (20) and DnaJ (27), respectively, i.e. close to 48 °C. These midpoint temperatures are lower than those indicated by the circular dichroism measurements (Figs. 2A and 3A) and infrared spectroscopy (27). We also cannot exclude that the observed decrease in circular dichroism is due in part to interactions of the proteins with the surface of the cuvette.

GrpE is the only constituent of the DnaK chaperone system that undergoes extensive structural alterations within the physiologically relevant temperature range. In both circular dichroism measurements and differential scanning calorimetry, the first of the two thermal transitions becomes evident at ~35 °C and reaches its midpoint at 48 °C (Figs. 4 and 5). This low-temperature transition is fully reversible. Because we did not observe a concentration-dependent shift of the first transition in GrpE, we assume that this transition is not accompanied by a change in the state of oligomerization. This notion is confirmed by the observation that the maximum rates of the GrpE-catalyzed R → T conversion are always reached at ~40 °C, irrespective of the concentration of GrpE (see below). A single unfolding transition in GrpE has been reported previously (28). The circular dichroism measurements in that study were performed up to a temperature of ~80 °C, whereby the second thermal transition may not have become apparent.

In accord with the thermal stability of DnaK (Fig. 2) and DnaJ (Fig. 3), the DnaJ-catalyzed T → R conversion of DnaK shows an Arrhenius temperature dependence within the physiologically relevant temperature range (Fig. 6A). The rates of increase in fluorescence of peptide a-ala-p5, which accompanies the conversion of DnaK from its low-affinity state to its high-affinity state, showed a similar temperature dependence (Fig. 6B). In contrast, the GrpE-induced R → T conversion substantially deviated from the Arrhenius temperature dependence (Fig. 7A). The same deviation from normal temperature dependence applied for the GrpE-triggered release of peptide (Fig. 7B) and the release of fluorescence-labeled nucleotide (Fig. 7C). However, the R → T conversion, unless triggered by GrpE, complied with the Arrhenius equation. The non-Arrhenius rate-temperature curve was only and always observed if the R → T conversion was catalyzed by GrpE; the deviant temperature dependence must thus be due to GrpE. The obvious sensitivity of the functionality of GrpE toward temperature correlates with the reversible structural transition of GrpE in the same temperature range. Experiments are underway to assign the thermal transitions to specific structural features of GrpE.

The decrease in efficacy of GrpE in catalyzing the ADP/ATP exchange at higher temperatures may be due to either a decreased affinity for DnaK or a decreased specific activity. Dimeric GrpE forms a tight complex with DnaK; at ambient temperature, the dissociation equilibrium constant of the GrpE/DnaK complex is estimated to be 1 nM in the absence of nucleotide and 0.22 μM in the presence of MABA-ADP (29). Varying the concentration of GrpE (0.1–1 μM) does not shift the temperature at which the rate of nucleotide exchange is at its maximum, indicating that the stability of GrpE/DnaK complexes is not significantly impaired at higher temperatures. The crystal structure of GrpE complexed with the ATPase domain of DnaK (22) shows several noncontiguous contact areas between DnaK and GrpE, with the two largest being in the β-sheet domain of GrpE. Contact areas are also located at the COOH-terminal end of the long helix of GrpE (Fig. 1B). Temperature-dependent changes in these contact areas of GrpE might underlie its temperature-sensitive functional behavior. However, limited temperature-dependent structural changes in DnaK itself that might modulate its interaction with GrpE cannot be excluded. An exposed, conserved loop in the ATPase domain of DnaK is needed for stable interaction with GrpE, as has been shown by deletion of this loop (30). Besides, there might be an additional interaction of the extended helix of GrpE with the peptide-binding domain (22). However, there is no evidence for a significant thermal transition in DnaK in the physiologically important temperature range. In view of the thermal transition of GrpE, which is extensive, occurs in the relevant temperature range, and is reversible, GrpE seems to
the prime candidate to control the kinetics of the R → T conversion of DnaK in a temperature-dependent manner.

The differential temperature dependence of the DnaJ-de-
dependent T → R conversion and the GrpE-dependent R → T
conversion leads, with increasing temperature, to a progressive
shift of DnaK toward its high-affinity R state. This shift be-
comes particularly prominent at heat-shock temperatures due
to the decrease in the rate of the R → T conversion. In our
experimental setup, i.e. equimolar concentrations of DnaK,
DnaJ, and GrpE, the changes in interconversion rates between
15 °C and 48 °C indicate a 10-fold shift in favor of the R state of
DnaK at heat-shock temperatures (from 0.7% to 7%; Table I).
The fraction of R-state DnaK (7% of the total) seems modest.
However, together with the increase by more than 2 orders of
magnitude in the rate of ATP hydrolysis, i.e. of the T → R
conversion, observed in the presence of a protein substrate (11),
the shift might result in the sequestering of protein substrates
at heat-shock temperatures. During heat shock, DnaK and
DnaJ have indeed been reported to cooperatively retain ther-
masely unfolded substrate protein in a folding competent state
both in vivo (31, 32) and in vitro (31, 33), whereas GrpE is
required for the reactivation of the substrate protein after the
heat shock (31, 33). The occurrence of a sequestering mecha-
nism at heat-shock temperatures does not preclude the possi-
bility of an additional mechanism of action in which DnaK uses
the energy of ATP hydrolysis to exert conformational work
upon polypeptide substrates that have undergone off-pathway
folding (10, 34).

GrpE homologs exist in bacteria, eukaryotic mitochondria,
and chloroplasts, but not in the eukaryotic cytosol and the
endoplasmic reticulum. Whereas apparently not all Hsp70 sys-
tems depend on a separate nucleotide exchange factor, GrpE is
essential for bacterial viability at all temperatures (35). A
mutant of DnaK, which exhibits impaired interaction with
GrpE (30), has only marginal chaperone activity (36). In vitro,
efficient refolding of firefly luciferase requires GrpE in addition
to DnaK and DnaJ (7, 31). The yield of chaperone-assisted
refolding of firefly luciferase attains a maximum at a specific
molar ratio of GrpE to DnaJ and DnaK (29). Obviously, the
balance of ATP hydrolysis and nucleotide exchange, acceler-
ated by DnaJ and GrpE, respectively, and thus the ratio of
T-state to R-state DnaK, are important for effective refolding of
denatured proteins. The amount of the components of the
DnaK heat-shock system is known to be controlled through the
regulation of transcription, stability, and activity of $\text{grpE}$ (for a
review, see Ref. 12). The differential temperature dependence
of the T → R and R → T conversions described in this report

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**Table I**

| Temperature (°C) | $k_{\text{obs}, \text{R} \rightarrow \text{T}}$ (s$^{-1}$) | $k_{\text{obs}, \text{T} \rightarrow \text{R}}$ (s$^{-1}$) | (% of total DnaK) |
|-----------------|---------------------------------|---------------------------------|-----------------|
| 15              | 1.9                             | 0.013                           | 0.7             |
| 48              | 8.8                             | 0.62                            | 7               |

The values are taken from Fig. 7A.

The values are taken from Fig. 6A.
might be the basis of a mechanism for adapting the DnaK/DnaJ/GrpE chaperone system to heat-shock conditions by a modulation of functionality rather than by a regulation of quantity.

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