**Thermosensor Action of GrpE**

**THE DnaK CHAPERONE SYSTEM AT HEAT SHOCK TEMPERATURES**

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Temperature directly controls functional properties of the DnaK/DnaJ/GrpE chaperone system. The rate of the high to low affinity conversion of DnaK shows a non-Arrhenius temperature dependence and above −40 °C even decreases. In the same temperature range, the ADP/ATP exchange factor GrpE undergoes an extensive, fully reversible thermal transition (Grimshaw, J. P. A., Jelesarov, I., Schönfeld, H. J., and Christen, P. (2001) J. Biol. Chem. 276, 6098–6104). To show that this transition underlies the thermal regulation of the chaperone system, we introduced an intersubunit disulfide bond into the paired long helices of the GrpE dimer. The transition was absent in disulfide-linked GrpE R40C but was restored by reduction. With disulfide-stabilized GrpE, the rate of ADP/ATP exchange and conversion of DnaK from its ADP-ligated high affinity R state to the ATP-ligated low affinity T state continuously increased with increasing temperature. Reduced GrpE R40C, the conversion became slower at temperatures >40 °C, as observed with wild-type GrpE. Thus, the long helix pair in the GrpE dimer acts as a thermosensor that, by decreasing its ADP/ATP exchange activity, induces a shift of the DnaK-substrate complexes toward the high affinity R state and in this way adapts the DnaK/DnaJ/GrpE system to heat shock conditions.

Cells respond to an increase in temperature by increased synthesis of heat shock proteins (Hsps).1 Molecular chaperone systems of the Hsp70 family prevent the formation of protein aggregates and facilitate the folding of nascent polypeptide chains and denatured proteins (for comprehensive reviews, see Refs. 1 and 2). DnaK, an Hsp70 homolog of *Escherichia coli*, binds peptides and segments of denatured proteins in extended conformation (3, 4) and cooperates with two cohort heat shock proteins: DnaJ, an Hsp40 homolog, and GrpE (5). The DnaK/DnaJ/GrpE chaperone system has been extensively studied in *vitro* at ambient temperatures (6–12). DnaK alternates between the high affinity R state and in this way adapts the DnaK/DnaJ/GrpE system to heat shock conditions.

* Experimental Procedures

**Materials**—The R40C mutation was introduced into the vector carrying the gene of wild-type GrpE (15) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primer pairs 5′-GGTTCTGCTGATGCTGCCTGATCCGTCGC-3′ and 5′-GCCGCGGTCAAGCTGAGAGAG-3′ for the introduction of the Q36C mutation, 5′-GCCGCGGTCAAGCTGAGAGAG-3′ for the Q36C mutation, 5′-GCCGCGGTCAAGCTGAGAGAG-3′ for the introduction of the R40C mutation, and 5′-GCCGCGGTCAAGCTGAGAGAG-3′ and 5′-GCCGCGGTCAAGCTGAGAGAG-3′ for the E58C mutation (replacements are underlined). GrpE was prepared as described elsewhere (15, 20). Nonreducing buffers were used to purify GrpE Q60C, GrpE R40C, and GrpE E58C. The yield of the preparations was ~130 mg protein/liter of cell culture. The disulfide-linked dimer was formed through air oxidation at a concentration of 250 μM GrpE in 50 mM Tris-HCl, 100 mM ADP/ATP exchange factor, DnaK is reconverted from the R state into the low affinity T state, releasing the substrate.

Heat shock proteins, by definition, are induced by a heat shock, i.e. a transient increase in temperature enhances the expression level of chaperones and co-chaperones. The transcription of the genes of DnaK and its co-chaperones DnaJ and GrpE is controlled by the initiation factor σ32 of RNA polymerase (for a recent review, see Ref. 13). Recently, we have investigated the direct effect of elevated temperatures on the isolated DnaK/DnaJ/GrpE chaperone system. GrpE, which is an elongated homodimer both in solution (14, 15) and in crystalline form (Fig. 1 and Ref. 16), has been found to undergo two well separated temperature-induced conformational transitions with midpoints at ~48 and 75–80 °C as evident from circular dichroism measurements and differential scanning calorimetry (17). The first transition, which occurs in the physiological temperature range, has proven to be fully reversible. A similar study of the nucleotide exchange factor from *Thermus thermophilus*, has confirmed the occurrence of a reversible thermal transition in GrpE (18). The low temperature transition has been tentatively ascribed to different parts of GrpE. In GrpE from *T. thermophilus*, the first transition (at 90 °C) was attributed to the χ-domain (Fig. 1 and Ref. 18); in the case of *E. coli* GrpE, based on truncation experiments that decrease the stability of the GrpE dimer, the low temperature transition was ascribed to the long helix pair (Fig. 1 and Ref. 19). The reversible low temperature conformational transition appears to be of functional importance (17). The transition correlates with a negative deviation from Arrhenius temperature dependence of the rate of the R → T conversion, the deviation continuously widening with increasing temperature. However, the structural transition and the functional changes have not been linked conclusively. Here, we have stabilized the paired NH2-terminal long helices in the GrpE dimer by introducing an intersubunit disulfide bond at the NH2-terminal end of the long helices (R40C; Fig. 1). Our results show that GrpE, in particular its pair of long helices (residues 40–87), acts as a thermosensor in the temperature-dependent tuning of the DnaK/DnaJ/GrpE heat shock system.

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‡ The abbreviations used are: Hsp, heat shock protein; MABA-ADP, 5′-(N′-methylanthraniloylamino)butyl)-8-aminoadenosine 5′-diphosphate.
NaCl, pH 8.5, for ~10 h at 25 °C. For circular dichroism measurements and differential scanning calorimetry, GrpE was dialyzed against 25 mM potassium phosphate, pH 7.0, for 18 h. To reduce GrpE R40C, 1 mM 1,4-dithiothreitol was added to the buffer 1 h before starting the experiment. The concentration of GrpE was determined photometrically with a molar absorption coefficient of ε280 = 2,720 M⁻¹ cm⁻¹. Throughout this report concentrations of GrpE refer to the monomer. DnaK was purified as described elsewhere (7, 11, 21). Its concentration was determined photometrically with a molar absorption coefficient of ε280 = 14,500 M⁻¹ cm⁻¹ (22). The molecular masses of the proteins were confirmed by mass spectroscopy.

Peptide ala-p5 (ALLLSAPRR) is a high affinity ligand for DnaK and was synthesized as described elsewhere (11). MABA-ADP was a gift from Dr. J. Reinstein (Max Planck Institut für Molekulare Physiologie, Dortmund, Germany) and had been synthesized as described elsewhere elsewhere (10). A fresh ATP stock solution (50 mM disodium salt, pH 7.0; Fluka) in a 0.52-ml volume was used. Technical details and performance of the instrument have been described elsewhere (24). The protein was dialyzed with a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a control, in reducing buffer. The SDS-PAGE broad range molecular weight standard (Bio-Rad) was used.

Circular Dichroism Measurements—Circular dichroism was measured with a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a thermostated cuvette with a 1- or 0.2-mm path length. The temperature was controlled with a programmable water bath. At fixed temperatures, four spectra between 250 and 185 nm (band pass, 2 nm) were recorded at a scan rate of 5 nm min⁻¹ and averaged. The time course of temperature-induced conformational changes was followed by continuously monitoring the ellipticity at 222 nm (band pass, 2 nm) at a scan rate of 1 °C min⁻¹.

Differential Scanning Calorimetry—A VP-DSC microcalorimeter (MicroCal, Northampton, MA) equipped with twin coin-shaped cells of 0.52-ml volume was used. Technical details and performance of the instrument have been described elsewhere (24). The protein was dialyzed for 18 h against the same batch of buffer that was used to establish the baseline (25 mM potassium phosphate, pH 7.0). Instead of degassing the sample, two successive prescan cycles of heating and rapid cooling were performed between 5 and 35 °C. The scanning rate was 1 °C min⁻¹. The data were corrected for the buffer-buffer base line and normalized for the concentration.

**RESULTS**

**Introduction of the Intersubunit Disulfide Bond Stabilizes GrpE**—SDS-PAGE showed that GrpE R40C exists as a disulfide-stabilized dimer under nonreducing conditions (Fig. 2). No species other than the dimer was detected. The formation of the covalent dimer of GrpE R40C was confirmed with mass spectrometry (not shown). Under reducing conditions, GrpE R40C migrated to the same position as wild-type GrpE, indicating complete transformation into a monomeric species at the denaturing conditions of SDS-PAGE. Wild-type GrpE, which does not possess any cysteine residues, under both reducing and nonreducing conditions, migrated to a position corresponding to a monomeric species.

Thermally induced changes in the structure of GrpE were monitored with circular dichroism spectroscopy and differential scanning calorimetry. The circular dichroism spectra of GrpE R40C under nonreducing and reducing conditions (Fig.
A) and of wild-type GrpE (17) were identical at 15 °C, indicating that neither the introduction of the cysteine residue nor the formation of the disulfide bond affected the structural integrity of the GrpE dimer. Analogous to wild-type GrpE, GrpE R40C under reducing conditions underwent two well separated thermal transitions with midpoints at 48 and 75–80 °C, as indicated by circular dichroism thermal unfolding curves (Fig. 3B). Similar to wild-type GrpE, a substantial fraction of helicity was lost at 60 °C, and virtually no helicity was apparent at 95 °C (Fig. 3A). The unfolding proved reversible up to a temperature of 60 °C. In contrast, the temperature-induced unfolding of the oxidized, i.e. disulfide-stabilized, GrpE R40C dimer showed a single thermal transition with a midpoint at −72 °C as indicated by both circular dichroism measurements (Fig. 3B) and differential scanning calorimetry (Fig. 4). The transition at 48 °C was completely shifted to higher temperatures. Accordingly, at 60 °C substantially more helicity was observed in the circular dichroism spectrum, but again no helicity was observed at 95 °C (Fig. 3A). Wild-type GrpE and reduced GrpE R40C lose a significant fraction of ellipticity below 60 °C (60% of total), which accounts for 25% of the total heat absorbed. The exact position of the introduced disulfide bond proved to be crucial for effective stabilization of the helices (Table I). If the disulfide bond was placed four residues before the helices (Q36C; Fig. 1), only slight stabilization was obtained. When the disulfide bond was close to the middle of the long helices (GrpE E58C), the midpoint of the transition at lowest temperature was at 72 °C; however, the transition was considerably less cooperative (not shown) than in GrpE R40C.

Temperature Dependence of the Rates of GrpE-catalyzed R → T conversion—We examined the effect of temperature on the rate of the GrpE-mediated conversion of DnaK from its high affinity R state to its low affinity T state. The rate of the conversion was determined at fixed temperatures within the physiologically relevant range during a stepwise increase in temperature from 15 to 48 °C. Two different types of measurements were performed: (i) DnaK possesses a single tryptophan residue at position 102, which allows fluorescence spectroscopic monitoring of conformational changes including those accompanying the R → T conversion (6, 11, 26, 27) and (ii) fluorescence-labeled ADP allows to monitor the release of the nucleotide, the rate-determining step in ADP/ATP exchange, which underlies the R → T conversion (10, 17). With both reduced and oxidized GrpE R40C, the rates of the R → T conversion of DnaK at 25 °C, followed by the decrease in either intrinsic fluorescence of DnaK or fluorescence of MABA-ADP, were similar to the rates that had been measured with wild-type GrpE. The rate of the spontaneous R → T conversion in the absence of GrpE is slower by 2 to 3 orders of magnitude (Table II). With wild-type GrpE, the rate of the R → T conversion deviates from an Arrhenius temperature dependence by increasing progres-
TABLE I
Thermal stability of disulfide-linked GrpE dimers

| Construct     | Transition midpoint T_m °C |
|---------------|---------------------------|
|               | Reducing                  | Nonreducing               |
| GrpE Q36C     | 51                        | 53                        |
| GrpE R40C     | 48                        | 73                        |
| GrpE E58C     | 52                        | 72                        |

TABLE II
R → T conversion activity of GrpE R40C at low temperature

| Detection                  | GrpE R40C | Wild-type GrpE | Without GrpE |
|----------------------------|-----------|----------------|--------------|
| Observed rate k_obs s⁻¹    |           |                |              |
| Reduced                   | 3.3       | 4.7            | 0.02         |
| Oxidized                  | 3.3       |                |              |

In this study, we established the causal connection between the reversible thermal transition in GrpE and the GrpE-mediated temperature-dependent modulation of the DnaK-substrate interaction. The introduction of an intersubunit disulfide bond (R40C) into the GrpE dimer allowed us to study the R → T conversion of DnaK as a function of temperature with GrpE in either its disulfide-stabilized form or its reduced, nonstabilized form. The rate of the R → T transition of DnaK in the presence of the disulfide-stabilized GrpE R40C dimer did not decrease at temperatures above 40 °C as it did in the case of GrpE R40C with reduced disulfide bond or wild-type GrpE (Fig. 5). The data unequivocally show that GrpE is responsible for the temperature-dependent control of the functionality of the DnaK system and that the pair of long helices in the GrpE dimer is its thermosensor.

How is the melting of the helix pair communicated to DnaK? In disulfide-linked GrpE, the low temperature transition was completely absent by the criteria of both circular dichroism and differential scanning calorimetry (Figs. 3 and 4). Thus, the melting of the helix pair partakes in the low temperature transition, which is shifted to higher temperatures by the introduction of the disulfide bond. The crystal structure of the GrpE dimer complexed with the ATPase domain of DnaK (16) shows several noncontiguous contact areas, with the two largest contact areas being part of the β-sheet domain of GrpE (Fig. 1). However, contact areas are also located at the COOH-terminal end of the long helix pair. GrpE interacts with both lobes of the ATPase domain and appears to facilitate nucleotide exchange by stabilizing the open conformation of the ATPase domain (16). GrpE is assumed to force the nucleotide binding cleft of the ATPase domain of DnaK into an open conformation, thereby facilitating nucleotide exchange.

DISCUSSION

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The midpoint of the transition at the lowest temperature T_m of GrpE with introduced cysteine residues was determined under reducing and nonreducing conditions by circular dichroism spectroscopy. The changes in ellipticity at 222 nm were monitored. The midpoint of transition was determined by derivation of the unfolding curves. For GrpE R40C, the values are from Fig. 3. The midpoint of the transition at the lowest temperature in wild-type GrpE is at 48 °C (17).

The rates of the GrpE-induced R → T conversion of DnaK (1 μM) at 25 °C, as measured by monitoring either the decrease in intrinsic fluorescence of DnaK or the decrease in MABA-ADP fluorescence, are given for GrpE R40C (1 μM) under reducing and nonreducing conditions. The values for wild-type GrpE (1 μM) and for the spontaneous conversion in the absence of GrpE are taken from Ref. 17. For details, see “Experimental Procedures.”

The midpoint of the transition at the lowest temperature in wild-type GrpE is at 48 °C (17).
of the GrpE-DnaK complex is thus consonant with our data that show that the intact long helix pair of the GrpE dimer is of crucial importance for its nucleotide exchange activity. Melting of the helices results in a decreased efficacy of GrpE in catalyzing the ADP/ATP exchange. Melting of the helix pair might either affect the affinity of GrpE for DnaK or decrease the nucleotide exchange activity of GrpE. The latter explanation seems more likely because varying the concentration of GrpE (0.1–1 μM) did not shift the temperature at which the rate of nucleotide exchange is at its maximum, indicating that the affinity of GrpE for DnaK is not significantly impaired at higher temperatures (17). Recent structural investigations on GrpE and truncated derivatives thereof have indicated that the four-helix bundle may serve as a stable platform for the association of the long helices, which melt in the physiologically relevant temperature range (19). The stability of the NH2-terminal long helix pair was found to be linked to the presence of the COOH-terminal β-domains of GrpE, indicating that the melting of the long helix pair might be coupled with the DnaK-GrpE interaction, which is mediated by the β-domain (19). A structure-function study of GrpE of *T. thermophila* has revealed a reversible thermal transition in the physiological temperature range of the thermophilic organism (18). However, in this case the low temperature transition has been attributed to the globular COOH-terminal domain, with the pair of long helices melting at higher temperatures. Although the thermosensor functionality is attributed to a different structural element, it is remarkable that GrpE serves as a thermosensor in both a thermophilic archaean and a mesophilic bacterium (18).

To date it is unclear whether Hsp70 chaperone systems act either by passively sequestering apolar stretches of polypeptide chains or by exerting active conformational work upon the substrate (11). An analogous mechanistic alternative is being discussed for the Hsp70-mediated import of proteins into mitochondria (28). In the case of DnaK, the kinetic data on the rates of the T → R and R → T conversions indicate that the chaperone cycle is fast, particularly at high temperatures, at which the half-life of state R DnaK may be as short as ~10 ms (Table III). Thus, a model in which the DnaK/DnaJ/GrpE system holds the substrate protein sequestered for a prolonged period of time does not seem realistic. However, the increased ratio of the T → R to the R → T reaction rate at elevated temperatures, because of the thermal modulation of the GrpE activity, obviously leads to a shift of the steady state toward the high affinity R state (Table III) that amounts to a dynamic sequestering of the substrate. A more active role of the chaperone system in refolding polypeptide substrates is suggested by the fast rate of the chaperone cycle with a commensurately high ATP consumption. Above ~40 °C, the rate of the GrpE-mediated R → T conversion decreases with increasing temperature. At folding unfavorable temperatures, the (hypothetical) active role in refolding polypeptide substrates is apparently less important, whereas the substrates remain dynamically sequestered by DnaK through an even more pronounced shift of the steady state toward the high affinity R state. In conclusion, it may be argued that in the DnaK system both dynamic sequestering and active refolding work could occur, their relative extent being tuned by temperature.

GrpE is the only member of the DnaK/DnaJ/GrpE chaperone system that is essential for the survival of bacteria at all temperatures (29). In eukaryotic cells, a GrpE homolog is present in mitochondria, where it appears to be important for cell viability (30). Hsp70 chaperones have been divided into three subclasses on the basis of structural differences in the ATPase domain, which appear to underlie substantial differences in the rate of spontaneous nucleotide exchange (31). Only for the two subclasses with the slowest spontaneous nucleotide exchange rates (with the two representatives DnaK and Hsc70) are nucleotide exchange factors known to exist: GrpE, which is found in bacteria and mitochondria, and the Bag family of proteins, which is found in the eukaryotic cytosol and nucleus (for a review, see Ref. 32). DnaK has a particularly low rate of spontaneous nucleotide exchange; GrpE greatly accelerates the exchange. Even small disturbances in the kinetics of nucleotide release, and therefore of substrate release, caused by the introduction of single point mutations in the ATPase domain of DnaK, were found to considerably affect the chaperone activity of DnaK (31). Tuning of the GrpE-catalyzed R → T conversion, although the absolute value of GrpE is rather hypothetical. The proportion of R state DnaK was obtained from $t_{1/2,R} = t_{1/2,T} + t_{1/2,T} - r^3$. In *Salmonella*...

### TABLE III

| Temperature | GrpE activity | $t_{1/2,T}$ | $t_{1/2,R}$ | R state |
|-------------|---------------|-------------|-------------|--------|
| °C          | (obs./Arrh.)   | R state     | T state     | % of total DnaK |
| 15          | 1             | 37          | 750         | 5      |
| 37          | 0.74          | 7           | 44          | 13     |
| 42          | 0.55          | 6           | 30          | 16     |
| 48          | 0.25          | 8           | 16          | 33     |

* The activity of GrpE is given as the ratio of the observed rates for the GrpE-catalyzed R → T conversion (38, 39) and the extrapolated Arrhenius rates (Fig. 5C).

The temperature dependence of the half lives of R state and T state DnaK, extrapolated to in vivo conditions.
tion of activity was attributed to a temperature-sensitive equilibrium between monomer and coiled coil (34). Very little is known about the molecular basis of the physiology of thermo-reception by neurons. An ion channel of the TRP family has been found to be activated by heat (35), and recently a cold receptor has been identified as a member of the same family (36, 37). The mechanism of activation is not yet known. GrpE with an experimentally accessible structure, a straightforward functional assay, and central significance in cell viability seems an attractive system to investigate the thermosensor action of a protein.

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