Tuning of DnaK Chaperone Action by Nonnative Protein Sensor DnaJ and Thermosensor GrpE*

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DnaK, an Hsp70 molecular chaperone, processes its substrates in an ATP-driven cycle, which is controlled by the co-chaperones DnaJ and GrpE. The kinetic analysis of substrate binding and release has as yet been limited to fluorescence-labeled peptides. Here, we report a comprehensive kinetic analysis of the chaperone action with protein substrates. The kinetic partitioning of the (ATP-DnaK)-substrate complexes between dissociation and conversion into stable (ADP-DnaK)-substrate complexes is determined by DnaJ. In the case of substrates that allow the formation of ternary (ATP-DnaK)-substrate-DnaJ complexes, the cis-effect of DnaJ markedly accelerates ATP hydrolysis. This triage mechanism efficiently selects from the (ATP-DnaK)-substrate complexes those to be processed in the chaperone cycle; at 45 °C, the fraction of protein complexes fed into the cycle is 20 times higher than that of peptide complexes. The thermosensor effect of the ADP/ATP exchange factor GrpE retards the release of substrate from the cycle at higher temperatures; the fraction of total DnaK in stable (ADP-DnaK)-substrate complexes is 2 times higher at 45 °C than at 25 °C. Monitoring the cellular situation by DnaJ as nonnative protein sensor and GrpE as thermosensor thus directly adapts the operational mode of the DnaK system to heat shock conditions.

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family assist folding and refolding of nascent and stress-denatured proteins, assembly and disassembly of protein complexes, and translocation of polypeptide chains across membranes. All of these activities appear to rely on the transient interaction of Hsp70 with short hydrophobic segments of their protein substrates. The cycle of substrate binding and release is driven by the hydrolysis of ATP. DnaK, an Hsp70 homolog in Escherichia coli, consists of a 44-kDa NH2-terminal ATPase domain (1, 2) and a 25-kDa COOH-terminal substrate-binding domain (3). Hydrolysis of DnaK-bound ATP and ADP/ATP exchange control the functional properties of the substrate-binding domain (4). ATP-ligated DnaK (T-state DnaK) exhibits low affinity for substrates and fast rates of binding and release, whereas ADP-ligated (R-state) DnaK is characterized by high substrate affinity and slow kinetics (5, 6). DnaK acts in concert with two co-chaperones (Fig. 1). DnaJ, an Hsp40 homolog, stimulates the hydrolysis of ATP and thus promotes the formation of high affinity (ADP-DnaK)-substrate complexes, whereas GrpE facilitates the exchange of ADP for ATP and thus triggers the release of substrate from the cycle (7–9).

Fluorescence-labeled peptides have allowed the kinetic analysis of the formation of DnaK-peptide complexes (6). With protein substrates, however, no kinetic data have been reported to date, DnaK-protein complexes having solely been examined by size exclusion chromatography (5), by nondenaturing gel electrophoresis (2), and by measuring fluorescence anisotropy under steady-state conditions (10).

A major question in the field of molecular chaperones is how targeted chaperone action is selectively triggered. Protein substrates, such as firefly luciferase, αβ2 (9), denatured rhodanese, or RepA (11, 12), accelerate the DnaJ-stimulated hydrolysis of DnaK-bound ATP by 1–2 orders of magnitude. DnaJ not only stimulates the ATPase activity of DnaK but also associates with unfolded proteins and prevents protein aggregation (13–15). DnaJ has been suggested to select the chaperone substrates and then to transfer them to DnaK (9, 14, 15). However, no experimental evidence has been reported as yet for this substrate transfer. A plausible mechanism of the targeting action of DnaJ is provided by the concept of a cis-effect of DnaJ on DnaK in ternary (ATP-DnaK)-substrate-DnaJ complexes (11, 12); the simultaneous binding of DnaK and DnaJ to one and the same polypeptide chain results in a higher effective concentration of DnaJ.

During heat shock and other cellular stress, the expression level of DnaK and its co-chaperones is enhanced to cope with the rising amount of misfolded and aggregation-prone proteins (for a recent review, see Ref. 16). In E. coli, this well-known heat shock response is mediated by the transcription factor α32, which directs RNA polymerase to transcribe the particular set of heat shock genes. Within minutes after onset of the heat shock (temperature ≥42 °C), the cellular concentration of DnaK is increased about 2-fold (17, 18). Recent in vitro experiments with the isolated DnaK/DnaJ/GrpE chaperone system have indicated that the system also responds directly to heat shock conditions; a mere increase in temperature suffices to increase the fraction of peptide substrate sequestered by DnaK.
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FIGURE 1. The DnaK/DnaJ/GrpE chaperone cycle. The substrate S first associates with ATP-ligated T-state DnaK. DnaJ then feeds the substrate into the cycle by stimulating the hydrolysis of DnaK-bound ATP. In the high affinity R state, the substrate remains bound to DnaK, the dissociation rate being too slow to be of physiological significance. GrpE releases the substrate from the cycle by facilitating ADP/ATP exchange. The fraction of high affinity R-state DnaK, and thus of sequestered substrate, is controlled by the conjoint action of the co-chaperones DnaJ and GrpE.

(19). This effect is due to the differential temperature dependence of the activities of the two co-chaperones (20). At heat shock temperatures, GrpE undergoes a fully reversible conformational transition, which decreases its nucleotide exchange activity (20–25). In contrast, DnaK and DnaJ do not show any conformational changes between 15 and 48 °C.

Here, we report the kinetics of binding, release, and dynamic sequestering of protein substrates in the DnaK chaperone cycle under normal and heat shock conditions. Our results show that the cis-effect of DnaJ in ternary (ATP-DnaK)-substrate-DnaJ complex acts as a triage mechanism selectively feeding non-native proteins into the chaperone cycle. DnaJ as nonnative protein sensor and GrpE as thermosensor directly adapt the operational mode of the chaperone system to heat shock conditions.

EXPERIMENTAL PROCEDURES

Materials—The H541C mutation was introduced into the gene of wild-type (WT) DnaK in plasmid pTPG9 (a gift from Dr. C. Georgopoulos and Dr. D. Ang, Geneva, Switzerland) with the QuiKChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The nucleotide replacements were verified by DNA sequence analysis. WT DnaK and DnaK H541C were purified as described previously (26). The concentration of WT DnaK was determined photometrically with the molar absorption coefficient $\varepsilon_{280} = 14,500 \text{ M}^{-1} \text{cm}^{-1}$ (27). The stock solution of WT DnaK was stored in assay buffer (25 mM Hepes/NaOH, 100 mM KCl, 10 mM MgCl$_2$, pH 7.4) at $-80^\circ \text{C}$. For fluorescence labeling, 760 $\mu$M DnaK H541C in 25 mM Hepes/NaOH, 100 mM KCl, 10 mM MgCl$_2$, pH 7.0, was reacted with a 5-fold molar excess of acrylodan in the presence of 10 mM ATP (to prevent labeling at Cys$^{15}$ located in the ATPase domain) for 3 h at room temperature. The acryl group of acrylodan served to attach the environmentally sensitive fluorophore covalently to the sulfhydryl group of Cys$^{541}$. The reaction mixture was filtered (0.2 $\mu$m); acrylodan-labeled DnaK H541C (a-DnaK H541C) was purified by size exclusion chromatography (Fractogel EMD BioSec 650 S from Merck) in 25 mM Tris/HCl, 1 mM EDTA, pH 8.0, containing 10 mM 2-mercaptoethanol, transferred into assay buffer with a Sephadex G-25 PD-10 column (Amersham Biosciences), and stored at $-80^\circ \text{C}$. Incorporation of a single fluorophore per DnaK H541C molecule was confirmed by mass spectrometry. The concentration of the stock solution was determined with the Bradford method (28) with bovine serum albumin as a standard. DnaJ and GrpE (gifts from Dr. H.-J. Schönhoff, Hoffmann-La Roche, Basel, Switzerland) were prepared as reported elsewhere (29, 30). The stock solutions in 50 mM Tris/HCl, 100 mM NaCl, pH 7.7, were kept at $-80^\circ \text{C}$. The concentrations of DnaJ and GrpE were determined photometrically with $\varepsilon_{277} = 18,100 \text{ M}^{-1} \text{cm}^{-1}$ and $\varepsilon_{279} = 2,720 \text{ M}^{-1} \text{cm}^{-1}$, respectively. Throughout this report, the indicated protein concentrations refer to the respective protonomer. Bacteriophage P1 RepA (the expression strain was a gift from Dr. D. Chatteraj, NCI, National Institutes of Health, Bethesda, MD) was expressed and purified as described (31). The concentration of the stock solution in assay buffer was determined with the Bradford method with bovine serum albumin as a standard. Bovine $\alpha$-lactalbumin (type III) was purchased from Sigma. For permanent denaturation, $\alpha$-lactalbumin (850 $\mu$M) was dissolved in 6 M guanidine hydrochloride, 0.5 M Tris/HCl, 5 mM EDTA, pH 8.6, containing 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Pierce) as reducing agent and incubated for 1 h at 37 °C. The sulfhydryl groups of the 8 cysteine residues were reacted with 500 mM iodoacetamide (Sigma) for 5 min at 25 °C. The reaction was stopped with 1 M 2-mercaptoethanol, and the denatured protein was transferred into 3 M guanidine hydrochloride, 20 mM Tris/HCl, pH 7.4, with a PD-10 column and stored at $-80^\circ \text{C}$. The molecular mass of reduced and carboxymethylated $\alpha$-lactalbumin (RCMLA) was confirmed by mass spectrometry, and the concentration (420 $\mu$M) of the stock solution was determined with the Bradford method with bovine serum albumin as a standard. Peptides pp (CALLQSRLLLSAPRRAATARA) and p4 (CALLQSRLLS), both derivatives of the prepiece of chicken mitochondrial aspartate aminotransferase, were custom-synthesized by Chiron, Australia, or synthesized by Dr. S. Klauser in our Institute with an ABI 430A Peptide Synthesizer (Applied Biosystems). The concentration of the stock solution of pp in 1 mM dithiothreitol was determined by amino acid analysis. Peptide p4 was acrylodan-labeled and purified as described (19). The concentration of the acrylodan-labeled peptide p4 (a-p4) stock solution in 30% (v/v) acetonitrile was determined photometrically with $\varepsilon_{380} = 20,000 \text{ M}^{-1} \text{cm}^{-1}$ (Molecular Probes, Inc., Eugene, OR). ATP-Na$_2$ (purity >98%) and ADP-Na$_3$ (purity >90%) were purchased from Fluka. N8-(4-N'-methylanthraniloylaminobutyl)-8-aminoadenosine-5'-diphosphate (MABA-ADP; a gift from Dr. J. Reinstein, Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany) had been synthesized as described (32). [2,5,8-3H]Adenosine-5'-triphosphate ammonium salt (37.0 Ci/mmol) was from Amersham Biosciences.

Equilibrium Fluorescence Measurements—A PerkinElmer Life Sciences spectrofluorometer LS50B, equipped with a stirrer and a thermostated cuvette holder, was used to record fluorescence emission spectra of a-DnaK H541C. The excitation wavelength was set at 370 nm (band pass 4 nm), and the spectra were recorded from 400 to 600 nm (band pass 4 nm). The dissociation equilibrium constants ($K_d$) of a-DnaK H541C for RepA, RCMLA, or pp were determined by fluorescence titration. The $K_d$ values were obtained from least squares fits of the fluores-
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cence emission intensity of a-DnaK H541C at 500 nm (F) as a function of substrate concentration to the equation,

$$F = F_0 + PL \cdot \frac{(F_{\text{max}} - F_0)}{P_I} = F_0 + \frac{((F_{\text{max}} - F_0)/2)(K_d + L_I + P_I)}{((K_d + L_I + P_I)^2 - 4P_I \cdot L_I)^{0.5}} \quad \text{(Eq. 1)}$$

where $PL$ represents the concentration of a-DnaK H541C-substrate complex, $P_I$ is the total concentration of a-DnaK H541C, $L_I$ is the total substrate concentration, $F_0$ is the emission intensity of a-DnaK H541C in the absence of substrate, and $F_{\text{max}}$ is the emission intensity in the presence of substrate at saturating concentrations. All experiments were performed in assay buffer at a volume of 800 µl in a 1 × 0.4-cm cuvette. Fluorescence intensity and concentration of substrate were linearly corrected for the increasing sample volume during titration. Analogous calculations based on the difference in area between 440 and 600 nm gave virtually the same $K_d$ values.

Fast Kinetic Fluorescence Measurements—An SX18 MV stopped-flow apparatus from Applied Photophysics served to record changes in the fluorescence emission intensity of a-DnaK H541C, a-p4, and MABA-ADP. a-DnaK H541C and a-p4 were excited at 370 nm (band pass 4.5 nm), and MABA-ADP was excited at 360 nm (band pass 5.0 nm). The emitted light passed through a high pass filter with a 455-nm cut-off. The temperature of the syringes and the cuvette was controlled with a circulating water bath. The temperature of the cuvette was controlled with a circulating water bath (±0.5 °C). The solutions were equilibrated for at least 3 min at the respective temperature before starting the reaction by mixing 70 µl of each solution. All experiments were performed in assay buffer. Reaction traces of at least four measurements were averaged. The second-order binding rate constants ($k_1$) and the dissociation rate constants ($k_{-1}$) of a-DnaK H541C for RepA, RCMLA, or pp were obtained from least squares fits of the observed pseudo-first order rate constant of complex formation ($k_{\text{obs}}$) as a function of substrate concentration to the equation, $k_{\text{obs}} = k_1 \cdot [\text{substrate}] + k_{-1}$. The dissociation constant of the first step in complex formation ($K_{\text{d1}}$) was calculated as $K_{\text{d1}} = k_{-1}/k_1$. $K_{\text{d1}}$ should not be mistaken for the overall dissociation equilibrium constant $K_p$ which was determined by fluorescence titration.

Single-turnover ATPase Activity—To perform ATP-DnaK complexes, DnaK was incubated with excess radioactively labeled ATP for 10 min at 4 °C. DnaK-bound ATP was separated from free ATP by rapid size exclusion chromatography with a Sephadex G-50 NICK column (Amersham Biosciences) in assay buffer at 4 °C as described (33). DnaJ was preincubated in assay buffer in the absence and presence of RepA, RCMLA, or pp for 3 min at the indicated temperature. ATP hydrolysis was started by the addition of ATP-DnaK complex to a final volume of 50 µl. The reaction was quenched at certain time points by mixing 4 µl of the sample solution with 4 µl of formic acid. ADP was separated from ATP on poly(ethyleneimine)-cellulose thin layer plates (Merck) with 1 m formic acid, 0.7 m lithium chloride as eluant. ATP and ADP were quantified by liquid scintillation counting. A least squares fit of the increase in ADP concentration with time to a single-exponential equation gave the rate constant of ATP hydrolysis.

Steady-state ATPase Activity—Test tubes containing DnaK, DnaJ, and GrpE in assay buffer were preincubated in the absence and presence of RepA, RCMLA, or pp for 3 min at the indicated temperature. ATP hydrolysis was started by the addition of 10 µM (or 100 µM in the case of RepA) radioactively labeled ATP. The final volume of the sample solution was 50 µl. The production of ADP was followed as described above. In all assays, the fraction of ATP hydrolyzed did not exceed 20% of the initial concentration. The extent of spontaneous ATP hydrolysis during the assay was negligible.

RESULTS

We determined the kinetics of the following phases in the DnaK/DnaJ/GrpE chaperone cycle (Fig. 1): (i) binding and release of protein substrates to and from ATP-DnaK by real-time recording of changes in the fluorescence of a-DnaK H541C; (ii) T → R conversion, including the cis-effect of DnaJ on the (ATP-DnaK)-protein complexes; and (iii) R → T conversion, including the thermosensor effect of GrpE. All assays were run at 25, 37, and 45 °C. The concentrations of the chaperones were set at 500 nM DnaK, 100 nM DnaJ, and 200 nM GrpE in accord with the estimated concentration ratios of DnaK/DnaJ/ GrpE = 5:1:2 in the cell (18, 34).

Fluorescence-labeled a-DnaK H541C—To label DnaK with the thiol-reactive, environmentally sensitive fluorophore acrylodan, we introduced an additional cysteine residue in DnaK by
amino acid substitution. Because DnaK has only one cysteine residue, which is located in its ATPase domain and is easily blocked with ATP (35), any new cysteine residue, if in an accessible position, can be specifically labeled. We tested several positions (i.e. His541 (35), Met469 (36), Glu430, and Asn463) that are located near the substrate-binding cavity of DnaK for attaching the fluorescent probe. Based on the criteria of fluorescence signal quality, maintained ATPase activity and chaperone efficacy in the luciferase refolding assay, we chose a-DnaK H541C for experimentation (Fig. 2). The molecular activity of a-DnaK H541C (500 nM) was the same as that of WT DnaK for ATP hydrolysis ($k_{cat} = 0.008 s^{-1}$) in the presence of 100 nM DnaJ and 200 nM GrpE under steady-state conditions at 25 °C. In the luciferase refolding assay, the same rate and yield was obtained with a-DnaK H541C as with WT DnaK (not shown). Moreover, the far-UV circular dichroism spectra of a-DnaK H541C and WT DnaK were almost identical (not shown).

We compared the complex formation of fluorescence-labeled a-DnaK H541C and peptide pp with the complex formation of WT DnaK and fluorescence-labeled peptide a-pp. The $K_d$ values of a-DnaK H541C for pp in the presence of ATP or ADP were determined by fluorescence titration (Fig. 3A). Binding of pp to a-DnaK H541C resulted in an increase in fluorescence emission intensity and a blue shift in the wavelength of maximum emission. The $K_d$ values calculated from the hyperbolic titration curves (Fig. 3B) were close to the previously determined values of WT DnaK for a-pp (Table 1). The second-order binding rate constant ($k_{i}$) and the dissociation rate constant ($k_{-i}$) of ATPa-DnaK H541C for pp were determined by measuring the observed pseudo-first order rate constant of complex formation ($k_{obs}$) as a function of pp concentration (Fig. 3C). The progress curves were best described with a double-exponential equation. The $k_{obs}$ value of the first phase (amplitude ~80% of total) was linearly proportional to the pp concentration (Fig. 3D). The derived values of $k_{i}$ and $k_{-i}$ were of the same order of magnitude as those of WT DnaK for a-pp (Table 1). The $k_{obs}$ value of the second, ~10 times slower phase of complex formation (amplitude ~20% of total) increased hyperbolically with increasing concentration of pp (not shown), suggesting that the rapid formation of an encounter complex is followed by a slower isomerization step. The similarity of a-DnaK H541C with WT DnaK regarding ATPase activity, performance in the luciferase refolding assay, and far-UV circular dichroism spectra as well as the similar values of the binding parameters of a-DnaK H541C for pp and WT DnaK for a-pp qualify a-DnaK H541C as a suitable means for investigating the interaction of DnaK with protein substrates. For monitoring the T → R and R → T conversions, however, a-DnaK H541C proved unsatisfactory, because the difference in the fluorescence signal between the two states under the chosen conditions was too small for reliable measurements.

**Complex Formation of a-DnaK H541C with Protein Substrates**—DnaK preferentially recognizes peptide segments comprising a hydrophobic core of four or five apolar amino acid residues and flanking regions with basic residues (37). Such protein segments typically reside in the interior of folded pro-
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TABLE 2
Rate constants (k<sup>a</sup> and k<sup>-1</sup>) and dissociation equilibrium constants for a-DnaK H541C-substrate complexes in the presence of ATP at different temperatures

| Substrate | k<sup>a</sup> in the presence of ATP<sup>a</sup> | k<sup>-1</sup> in the presence of ATP<sup>b</sup> | K<sub>d</sub> in the presence of ADP<sup>c</sup> |
|-----------|------------------------------------------|------------------------------------------|---------------------------|
| RepA      | 160,000 μM<sup>-1</sup>s<sup>-1</sup>   | 7.0 μM<sup>-1</sup>s<sup>-1</sup>   | 1.4 μM<sup>-1</sup> |
| RCMLA     | 120,000 μM<sup>-1</sup>s<sup>-1</sup>   | 3.7 μM<sup>-1</sup>s<sup>-1</sup>   | 3.2 μM<sup>-1</sup> |
| pp<sup>d</sup> | 880,000 μM<sup>-1</sup>s<sup>-1</sup> | 6.3 μM<sup>-1</sup>s<sup>-1</sup> | 12.1 μM<sup>-1</sup> |

<sup>a</sup> Determined by mixing the following solutions in a stopped flow apparatus: 500 nM a-DnaK H541C, 5 mM ATP plus substrate, 5 mM ATP. The concentration of substrate was varied between 2.5 μM and 12.5 μM. The reaction traces (Fig. 3C shows an example) were fitted with a double-exponential equation, and the observed rate constant (k<sub>obs</sub>) of the first phase was used to determine the values of k<sup>a</sup>, k<sup>-1</sup>, and K<sub>d</sub> (for details, see “Experimental Procedures”). The approximate calculated amplitudes of the first phase with RepA, RCMLA, and pp were as follows: at 25 °C: 55, 30, and 80% of the total amplitude, respectively; at 37 °C: 40, 60, and 75%, respectively; and at 45 °C: 30, 45, and 70%, respectively.

<sup>b</sup> The data for 37 °C are from Table 1.

<sup>c</sup> Determined by fluorescence titration of 200 nM a-DnaK H541C with substrate (0.25–30 μM) in the presence of 5 mM ATP. The titration curve with pp is shown as an example in Fig. 3A (for details, see “Experimental Procedures”).

<sup>d</sup> For comparison, K<sub>d</sub> values of (ADP-a-DnaK H541C)/substrate complexes are listed. The values were determined by fluorescence titration of 100 or 200 nM a-DnaK H541C with substrate (0.025–5 μM) in the presence of 1 mM ADP (for details, see “Experimental Procedures”).

DnaK-controlled T → R Conversion—The cis-effect of DnaJ on DnaK in ternary (ATP-DnaK)/substrate-DnaJ complexes accelerates ATP hydrolysis by 1–2 orders of magnitude (see the Introduction). We measured the DnaJ-stimulated ATPase activity of DnaK in the presence of RepA, RCMLA, or pp at 25, 37, and 45 °C. RepA appears to possess a DnaK-binding site and a separate binding site for DnaJ (40), whereas RCMLA binds only to DnaK (5), its affinity for DnaK being very low (13). Peptide pp binds to both chaperones (41) but is too short for forming ternary complexes. At both 37 and 45 °C, 2.5 and 5 μM RepA gave rise to the cis-effect of DnaJ and accelerated the rate of the DnaJ-stimulated ATP hydrolysis ~8 and ~15 times, respectively, under single-turnover conditions (Fig. 4A) (i.e. by the same factor as found earlier at 25 °C) (12). In contrast, RCMLA and pp, which are substrates that do not form ternary complexes and consequently do not elicit the cis-effect of DnaJ, only barely stimulated the rate of ATP hydrolysis at all temperatures tested.
We determined the rate of DnaJ-stimulated T → R conversion also by measuring steady-state ATPase activity. To ensure that the release of ADP and inorganic phosphate were not rate-limiting in the overall cycle, GrpE was added in a large excess. The rates determined under steady-state conditions, both in the presence and absence of RepA, proved to be similar to the single-turnover rates; in particular, the cis-effect of DnaJ found under single-turnover conditions is also observed in the steady state of the ATPase cycle. In the temperature range from 25 to 45 °C, the rate of ATP hydrolysis followed an Arrhenius temperature dependence (Fig. 4B). Likewise, although on a lower level due to the missing cis-effect of DnaJ, the rate of ATP hydrolysis in the presence of RCMLA and pp as well as in the absence of substrate increased exponentially (Fig. 4A). The rate of ATP hydrolysis at 25 °C was linearly dependent on the concentration of DnaJ from 0.1 to 1 μM measured by both single-turnover and steady-state ATPase activity assays (not shown).

Triage of (ATP-DnaK)-Substrate Complexes: Entering the Chaperone Cycle Versus Dissociation—Upon binding of substrate to ATP-ligated DnaK, the substrate either enters the chaperone cycle through DnaJ-stimulated T → R conversion of the complex or dissociates from DnaK into the solvent (Fig. 1). The fraction of substrate that is processed in the cycle is thus determined by the relative rates of these two alternative reactions. The cis effect of DnaJ (i.e. the acceleration of the T → R conversion in the presence of DnaJ plus protein substrate) resulted in a higher fraction of RepA fed into the chaperone cycle compared with the corresponding fractions of RCMLA and pp (Table 3). An increase in RepA concentration from 2.5 to 5 μM increased the fraction about 2-fold at all temperatures measured. With temperature increasing from 25 °C to 37 and 45 °C, an increasingly higher fraction of RepA was fed into the chaperone cycle due to the differential temperature dependence of the rate of the T → R conversion (Fig. 4) and the rate of dissociation of the (ATP-DnaK)-RepA complex (Table 2).

In contrast, the fractions of RCMLA and pp decreased with increasing temperature from 25 to 45 °C, due to the steep temperature dependence of their dissociation rates (Table 2). At 45 °C and 5 μM RepA, the fraction of RepA fed into the cycle was more than 1 order of magnitude higher than that of RCMLA or pp (Table 3).

GrpE-controlled R → T Conversion—The ADP/ATP exchange factor GrpE acts as thermosensor for the chaperone system (see the Introduction). We measured the GrpE-facilitated release of fluorescence-labeled MABA-ADP (32) in the presence of RepA, RCMLA, or pp at 25, 37, and 45 °C. The rate of MABA-ADP release proved to be substrate-independent at all temperatures (Fig. 5A). However, the measurement of the release of MABA-ADP does not include the complete R → T conversion; we therefore determined the rate of the R → T conversion also by the release of a-p4 upon conversion of high affinity (ADP-DnaK)-a-p4 into low affinity (ATP-DnaK)-a-p4 complex. The experiments were performed in the presence of saturating concentrations of inorganic phosphate, because the release of ADP from DnaK is markedly slowed down by inorganic phosphate (32, 42). The rate of the GrpE-facilitated release of a-p4, which was 1.41 s⁻¹ at 25 °C in the absence of inorganic phosphate, was indeed decelerated to 0.15 s⁻¹ in the presence of phosphate. The latter measurement corresponds to the conditions during the steady state of the chaperone cycle, in which inorganic phosphate is generated in situ by the hydrolysis of ATP. The rate of the GrpE-facilitated R → T conversion, both in the presence and absence of phosphate, deviated from Arrhenius temperature dependence by not being faster or even somewhat slower at 45 °C than at 37 °C (Fig. 5, A and B). The rate of MABA-ADP/ATP exchange at 25 °C was linearly dependent on the concentration of GrpE from 0.1 to 1 μM (not shown).

Increased Sequestration of Protein Substrate under Heat Shock Conditions—The fraction of substrate sequestered in high affinity R-state DnaK is conjointly controlled by DnaJ and GrpE (Fig. 1). While the rate of the DnaJ-stimulated T → R conversion increases exponentially with increasing temperature (Fig. 4B), the rate of the GrpE-facilitated R → T conversion increases less and less with increasing temperature and even decreases at
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temperatures above 40 °C (Ref. 20; see also Fig. 5B). The rates of the DnaJ-stimulated T → R conversion (Fig. 4) and the GrpE-facilitated R → T conversion (Fig. 5) served to calculate the fraction of total DnaK existing as high affinity (ADP-DnaK)-substrate complex in the case of RepA, RCMLA, or pp at 25, 37, and 45 °C (Table 4). Two effects were observed: first, the cis-effect of DnaJ resulted at all temperatures in a 5 and 9 times higher fraction of (ADP-DnaK)-RepA complex in the presence of 2.5 and 5 μM RepA, respectively, as compared with the fraction of high affinity R-state DnaK in the absence of substrate, whereas RCMLA and pp as substrates only slightly increased the fraction of R state at all temperatures; second, the thermosensor control of GrpE resulted in a fraction of R-state DnaK about twice as high at 45 °C as at 25 °C, the thermosensor effect being independent of the presence and absence of any substrate. Combined, the cis-effect of DnaJ (at 5 μM RepA) and the thermosensor control of GrpE (at 45 °C) increased the fraction of high affinity R-state DnaK by one order of magnitude over that with pp or RCMLA as substrates at 25 °C (Table 4).

DISCUSSION

Binding and Release of Protein Substrates—In this study, we explored the kinetics of the DnaJ/GrpE-controlled DnaK chaperone cycle with proteins, the physiological substrates, both under normal and heat shock conditions. To follow the complex formation of DnaK with protein substrates in real time, we labeled DnaK H541C with the thiol-reactive fluorophore acrylodan. The values of the kinetic and thermodynamic parameters of the formation of (ATP-a-DnaK H541C)-pp complexes correspond with those measured with unlabeled DnaK and a-pp. The two procedures for monitoring the interaction of DnaK with peptide substrates thus mutually validate one another. At all temperatures, the values of the binding parameters of ATP-a-DnaK H541C for RepA and RCMLA were found to be of the same order of magnitude as the values for pp and other DnaK-binding peptides reported previously (41). The values of $k_1$ and $k_{-1}$ obtained with short peptides are sequence-specific and lie in a relatively broad range; hence, the differences between the protein substrates RepA or RCMLA and the peptide substrate pp cannot be interpreted as reflecting a fundamental difference in complex formation of proteins and peptides with DnaK. Apparently, there is no significant steric hindrance for complex formation with these protein substrates and no notable interaction site other than the peptide-binding cleft. The DnaK-binding properties of a protein substrate are thus primarily defined by the amino acid sequence of the segment that interacts with the peptide-binding site of DnaK. This notion is consonant with the crystal structure (3) and solution structure (43) of the substrate-binding domain of DnaK, which was found to bind hydrophobic 7-residue segments of the substrate in an extended conformation.

Nonnative Protein Sensor DnaJ—Due to the cis-effect of DnaJ, which only becomes effective upon formation of ternary (ATP-DnaK)-substrate-DnaJ complexes, the fraction of RepA being fed into the chaperone cycle is substantially higher than that of RCMLA or pp. Neither RCMLA nor pp form ternary complexes, and therefore they cannot elicit the cis-effect of DnaJ. The kinetic partitioning between dissociation of the (ATP-DnaK)-substrate complex and its conversion to a high affinity (ADP-DnaK)-substrate complex provides a sorting mechanism for substrates. DnaJ acts as sensor for nonnative proteins; only proteins with at least two exposed hydrophobic stretches elicit its cis-effect and trigger the ATP-consuming chaperone action (Fig. 6). The sorting of (ATP-DnaK)-substrate complexes includes, of course, not only the de novo formed complexes but also T-state complexes generated from R-state complexes by ADP/ATP exchange, the alternative for these complexes being another round in the chaperone cycle or release into the solvent. The triage by DnaJ prevents the loading of the chaperone system with proteins or protein fragments that do not need or no longer need chaperone assistance and minimizes futile ATP consumption.

DnaJ and other Hsp40 that share the J domain essential for the interaction with Hsp70 proteins are found both as soluble and as membrane-anchored proteins. In contrast to the soluble Hsp40 homologs, the membrane-anchored homologs, such as DjlA in E. coli, Sec63p in yeast endoplasmic reticulum, and Mdj2p in yeast mitochondria, do not have a substrate-binding domain. In analogy to the substrate-binding domain of soluble Hsp40, which serves to form ternary complexes with nonnative protein substrates, anchoring of Hsp40 homologs to the membrane restricts the ATP-consuming chaperone action to the segment of the polypeptide chain emerging from the translocation channel. Similarly, uncoating of clathrin-coated vesicles might be triggered by bringing Hsc70 and auxilin (Hsp40) into close proximity upon attaining the distinct geometry of the completely assembled coat.

Thermosensor GrpE—The rate of GrpE-facilitated ADP/ATP exchange proved to be substrate-independent and only controlled by temperature. With increasing temperature, melting of the long helix pair in the GrpE dimer decreases the efficacy of GrpE in catalyzing nucleotide exchange (20, 21, 23, 24). At heat shock, the reduced activity of GrpE has recently been shown to increase the fraction of peptide substrate sequestered by DnaK (19) and indeed has proved advantageous for protecting protein substrates in vitro against both heat denaturation and aggregation (44).

The DnaK system in E. coli is not the only chaperone system that takes advantage of direct thermal control. GrpE in the thermophilic eubacterium Thermus thermophilus and Mge1p, a GrpE homolog in yeast mitochondria, have also been found to undergo a reversible thermal transition within the physiological temperature range (22, 25). Different from the melting of the helix pair in the E. coli GrpE dimer, the thermal transition in T. thermophilus GrpE affects the β-sheet domain and in Mge1p results in dimer dissociation. Direct adaptation to heat shock was also found in small heat shock proteins, which at heat shock temperatures undergo reversible conformational changes that improve their chaperone efficiency; responses of this kind have been reported for yeast Hsp26 (45), wheat Hsp16.9 (46), and rat Hsp22 (47) as well as for the endoplasmic reticulum-resident chaperone calreticulin (48). Hsp33 in E. coli is a redox-regulated chaperone, its activity being enhanced by reversible conformational changes upon oxidative stress (49).
Direct Heat Shock Response through Conjoint Action of DnaJ and GrpE—If cells are subjected to heat shock conditions, the concentration of nonnative proteins exposing hydrophobic binding sites for DnaK and DnaJ will increase. The rate of the DnaJ-stimulated ATP hydrolysis increases exponentially with increasing temperature and, in the presence of unfolded proteins, it is additionally accelerated by the cis-effect of DnaJ. The higher the concentration of nonnative proteins and thus the higher the concentration of ternary (ATP-DnaK)-substrate-DnaJ complexes, the more pronounced the cis-effect will be (compare the rates of the T → R conversion at 2.5 and 5 μM RepA) (Fig. 4). Hence, the fraction of (ATP-DnaK)-protein complexes converted into high affinity (ADP-DnaK)-protein complexes increases (Fig. 6). The temperature-induced decrease in the rate of GrpE-facilitated ADP/ATP exchange in its turn retards the release of the protein substrate from DnaK. DnaJ as sensor for nonnative proteins and GrpE as thermosensor thus conjointly promote dynamic sequestering of the protein substrate as high affinity complexes with ADP-DnaK, the ensuing decrease in concentration of free nonnative proteins preventing their aggregation.

The DnaK/DnaJ/GrpE chaperone machinery exerts two seemingly distinct functions (i.e. promoting protein folding and preventing protein aggregation). Both processes rely on a continuously running ATPase cycle. Due to the thermosensor control of GrpE, the overall rate of the DnaK ATPase cycle at 45 °C is similar to that at 37 °C (not shown). The steady-state R/T ratio of DnaK, however, is shifted toward the R state at heat shock temperatures. Apparently, sequestering of substrate by DnaK becomes more important at higher temperatures, which are unfavorable for folding. This temperature-dependent shift in the operational mode is consistent with the notion that the chaperone effect of the DnaK system might result from two different mechanisms (50–53): “active” folding assistance by performing conformational work on the substrate and “passive” sequestration of hydrophobic stretches of the polypeptide chain. Depending on temperature, the two modes might contribute to a varying degree to the chaperone effect. Nonnative protein sensor DnaJ and thermosensor GrpE adapt the operational mode of the DnaK system to the particular situation and optimize the chaperone effect. GrpE directly senses heat shock by a temperature-induced conformational change, whereas DnaJ indirectly senses heat shock or other cellular stress by the ensuing misfolded proteins.

Similar to the regulation of key enzymes in metabolic pathways, the chaperone action of the DnaK/DnaJ/GrpE system is thus not only controlled by changing the concentration of the chaperones but also by direct modulation of their activity. Moreover, and again in analogy to metabolic regulation, the instant and reversible response to elevated temperature bridges the time lag of the α32-mediated heat shock response and of its reversal.

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