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Physical interactions between members of the DnaK chaperone machinery: characterization of the DnaK.GrpE complex

Katherine L. Reid, Anthony L. Fink

Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064, USA

Abstract Many of the functions of the *Escherichia coli* Hsp70, DnaK, require two cofactors, DnaJ and GrpE. GrpE acts as a nucleotide exchange factor in the DnaK reaction cycle but the details of its mechanism remain unclear. GrpE has high affinity for monomeric native DnaK, with a K_d estimated at ≤ 50 nM. GrpE is a very asymmetric molecule and exists as either a dimer or trimer in its native state. The stoichiometry of GrpE to DnaK in the isolated complex was 3:1, suggesting a trimer. Formation of the complex is quite fast ($k_{on} > 1\ s^{-1}$), whereas the off-rate is very slow on the HPLC timescale ($k_{off} \leq 10^{-4}\ s^{-1}$). GrpE has no affinity for ATP or ADP, nor the oligomeric and molten globule states of DnaK. The complex is much more thermally stable than either GrpE or DnaK alone, and prevents the formation of the molten globule-like state of DnaK at physiologically relevant temperatures. Formation of the complex does not cause any change in secondary structure, as determined by the lack of change in the circular dichroism spectrum. However, binding of GrpE induces a similar tertiary structural change in DnaK to that induced by binding of ATP, based on the blue shift in λ_{max} from the fluorescence of the single tryptophan in DnaK. The nucleotide exchange properties of GrpE can be explained by the conformational change which may represent the opening of the nucleotide cleft on DnaK, subsequently inducing a low affinity state for ADP.

INTRODUCTION

The Hsp70 molecular chaperones have been implicated in essential cell functions under both normal and stress conditions. DnaK is a member of the 70 kDa heat shock protein family in *Escherichia coli* and plays a role in a variety of cellular reactions including protein folding (reviewed in Gething and Sambrook 1992). DnaK acts as a molecular chaperone by binding to extended polypeptide chains (Flynn et al 1991) and releasing them upon adenosine triphosphate (ATP) binding, in the presence of K^+ (Palleros et al 1993a). Many of DnaK's biological functions are regulated by two protein cofactors, DnaJ (41 kDa) and GrpE (23 kDa) (Georgopoulos et al 1992; Laloraya et al 1994). This is also the case for the Hsp70 family members in eukaryotic cells where DnaJ and GrpE homologues assist in at least some of the functions of Hsp70 (Cyr et al 1992; Bolliger et al 1994; Naylor et al 1995). GrpE and DnaJ work synergistically with DnaK in enhancing the rate of the reaction cycle by facilitating nucleotide exchange and stimulating the ATPase activity, respectively (Liberek et al 1991). It is important to examine the interactions of these regulators with DnaK to better understand the mechanism of the DnaK chaperone machinery.

DnaK consists of a 44 kDa N-terminal ATPase domain and a C-terminal domain containing the substrate protein-binding site (Chappel et al 1987). Small-angle X-ray scattering studies reveal the structure to be dumbbell-shaped (Wilbanks et al 1995; Shi et al 1996). GrpE has been shown to interact with DnaK via a conserved loop in the ATPase domain (Buchberger et al 1994). GrpE has a monomer molecular mass of 23 kDa, and has been

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Correspondence to: Prof. Anthony L. Fink, Tel: +1 408 459 2744; Fax: +1 408 459 2935; E-mail: enzyme@cats.ucsc.edu

reported to be non-globular in shape and to exist as a dimer (Schönfeld et al 1995). The DnaK reaction cycle involves a ternary complex of DnaK, adenosine diphosphate (ADP) and unfolded substrate protein, formed either from the binary ADP complex, or the binary ATP complex via interaction with DnaJ and subsequent hydrolysis of ATP. The next step involves nucleotide exchange, shown to be facilitated by GrpE, in which the ADP is replaced by ATP (Liberek et al 1991). The binding of ATP to DnaK leads to a conformational change and subsequent release of the substrate protein (Palleros et al 1993a).

The details of how GrpE stimulates the ATPase activity of DnaK, and the mechanism of its nucleotide exchange function in the DnaK reaction cycle are unclear. To further elucidate the role of GrpE in the reaction cycle of DnaK, we analyzed the conformations of DnaK.GrpE complexes using circular dichroism (CD), fluorescence and size exclusion gel filtration, as well as the stability of the complex compared with the individual components, and the effect of ATP and ADP on complex formation. Our results suggest that GrpE induces a similar conformational change in DnaK as seen in the presence of ATP which could account for its nucleotide exchange function.

MATERIALS AND METHODS

ATP refers to an equimolar solution of $MgCl_2$ and ATP (disodium salt, Sigma); ADP refers to an equimolar solution of $MgCl_2$ and ADP (monosodium salt, Sigma).

Protein purification

E. Coli DnaK was isolated and purified using the BL21-DE3/pET-11 expression system as previously described (McCarty and Walker 1991) with modifications as previously reported (Palleros et al 1993a). Purity was > 97% as determined by SDS-PAGE stained with Coomassie blue, and the DnaK was free of bound nucleotides. GrpE was expressed using the BL21-DE3/pET-11 expression system (a generous gift from John Flanagan, Brookhaven). Isolation of GrpE involved growing the cells to an OD of 0.4 in LB media containing 50 μg/ml carbenicillin. The cells were induced for 3 h using a final concentration of 1 mM isopropyl β-D thiogalactopyranoside (IPTG). Cells were spun for 10 min at 6000 rpm and the pellet was resuspended in 50 mM Tris, 10% sucrose, pH 8.0. Cells were lysed in the presence of 1 mg/ml lysozyme, 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0 and subsequently centrifuged for 60 min at 17 000 rpm. The protein was precipitated using 0.35 g/ml of ammonium sulfate and centrifuged again for 60 min at 17 000 rpm. The pellet was resuspended in the same buffer as used for lysis, excluding the lysozyme. The protein solution was dialyzed overnight against 1 liter of 4 M urea, 0.05 M sodium phosphate, 0.005 M Tris/HCl, pH 8.0. The protein solution was then poured over a DEAE column previously equilibrated in the same 4 M urea buffer. GrpE eluted between 0.4 M and 0.6 M KCl and was found to be > 97% pure as determined by SDS-PAGE stained with Coomassie blue.

Determination of the extinction coefficients of DnaK and GrpE

The molar extinction coefficient at 280 nm of both proteins was determined using three methods, Bio Rad Protein Assay (Bio Rad) and Micro BCA Protein Assay Reagent Kit (Pierce) and the method of Edelhoch as described by Pace et al (1995). Methods for the protein assays were performed as described by the manufacturer. Bovine serum albumin and gamma globulin were used as protein standards, giving an excellent linear correlation for both the Bio Rad and the BCA method. The molar extinction coefficients for DnaK and GrpE at 280 nm were determined to be 16 000 ± 1000 M^{-1} cm^{-1} and 1500 ± 100 M^{-1} cm^{-1}, respectively, with all three methods in close agreement.

Far-UV circular dichroism and fluorescence

CD spectra and thermal denaturations were performed as previously described (Palleros et al 1992). Fluorescence experiments were collected on a Perkin Elmer spectrophotometer model LS-5. Emission spectra were collected every 1 nm using an excitation at 280 nm, slit width 5 nm, and emission from 290 to 500 nm, slit width 3 nm. For the thermal unfolding experiments the temperature was increased at a constant rate of 0.33°C/min. using a Neslab temperature programmer. The protein concentrations and buffer conditions are given in the figure legends. Fluorescence kinetic experiments were monitored at 347 nm, using 3.7 μM GrpE and 2.0 μM DnaK, at 22°C. A baseline was obtained for samples of DnaK, DnaK/ATP, and DnaK/ADP at the observed λ_{max} values. To each of the samples a final concentration of 3.7 μM GrpE was added and the fluorescence intensity was observed. An emission scan was obtained at the completion of the reaction, subtracting out the contribution of 3.7 μM GrpE.

Size exclusion high performance liquid chromotography

High performance liquid chromotography (HPLC) experiments were carried out at 23 ± 1°C on a Spectraphysics UV3000 using a Bio-SEP 3000 silica column (600 × 7.8

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mm; Phenomenex). The mobile phase was 20 mM Tris, 200 mM KCl, pH 6.9; the flow rate was 1 ml/min; detection was by absorbance at 215 and 280 nm. The column was calibrated using a high molecular weight gel-filtration calibration kit (Pharmacia) and sodium azide. The size exclusion (SEC) partition coefficient, K_d, was calculated with the following equation $K_d = (V_i-V_o)/V_t$, where V_i is the elution volume of the protein, V_o is the void volume (11.2 ml), and V_t is the total solvent-accessible volume (24.0 ml).

Stoichiometry determination

Stoichiometry was determined as previously described (Palleros et al 1995). A 200 µl mixture of 23.8 µM GrpE and 15.2 µM DnaK were incubated for 15 min at 37°C and injected into the SEC HPLC. The center of the fraction containing the complex was collected at 0°C, and subsequently an aliquot was run on a SDS-PAGE Pharmacia Phast system using precast 8–25% acrylamide gels. The experiment was repeated using an excess of DnaK (23.8 µM) to GrpE (7.1 µM) to assure all GrpE was in complex. Determination of the response factor, defined as the relative Coomassie staining between different proteins, was performed using known control ratios of GrpE and DnaK and run on the same gel as the sample being analyzed. The areas of the bands corresponding to DnaK and GrpE on the gel were determined by densitometry using a gel scanner (ISCO model 1312) attached to an integrator (Spectraphysics, model 4270). The following equation was used to calculate the ratio of GrpE bound to DnaK under native conditions: [GrpE]/[DnaK] = $A_E{\cdot}k/A_D$ where: A_E =peak area of GrpE, A_D is the peak area of DnaK, and k is the response factor of protein to Coomassie staining.

ATPase activity

The ATPase activity of DnaK and GrpE was measured using thin layer chromatography (TLC) as described previously (Palleros et al 1993b). ATP and ADP were detected using a phosphorimager and analyzed using Image Quant Software. Typically concentrations were 0.01–1 µM DnaK, 1–10 µM GrpE, 5 µM DnaJ, 30 µM ATP, in 1 mM $MgCl_2$ 20 mM Tris, 200 mM KCl, pH 7.1. Carrier proteins were used with DnaK concentrations < 1 µM.

Cross-linking experiments

Glutaraldehyde (Sigma) cross-linking experiments were performed as described elsewhere (Creighton 1989) using 5, 10 and 15 µM GrpE. Subsequent to cross-linking, the protein was resolved on a 8–25% acrylamide precast gel and stained with Coomassie blue.

RESULTS

GrpE and DnaK form a very tight complex

The formation of a complex between DnaK and GrpE was readily distinguished by a unique peak in SEC HPLC of mixtures of the two proteins, even at submicromolar concentrations. The properties of this complex and its stability were examined by far UV-CD, fluorescence, SEC HPLC and SDS-PAGE. Since ATP and ADP play a large role in the regulation of the DnaK reaction cycle, the effects of these nucleotides on the rate of formation of the complex, its conformation and its stability were also investigated. GrpE alone had no ATPase activity. However, the intrinsic ATPase activity of DnaK, $k_{cat} = 9.8 \times 10^{-4}\ s^{-1}$ at 37°C, pH 7.0, was slightly enhanced in the presence of GrpE (5-fold molar excess), $k_{cat} = 2.0 \times 10^{-3}\ s^{-1}$. If DnaJ was also present (also 5-fold molar excess), then a substantial effect of GrpE on the ATPase activity of DnaK was observed, $k_{cat} = 2.6 \times 10^{-2}\ s^{-1}$. The results, that GrpE stimulates DnaK 2-fold, and in the presence of DnaJ, 25-fold, are in accord with previously reported activities (Liberek et al 1991). Evidence for the high affinity of GrpE for DnaK was found in the observation that changing the concentration of GrpE (over the 1–10 µM range) had no effect on the rate of ATP hydrolysis by DnaK (0.01 or 0.1 µM) and DnaJ (5 µM), i.e. the progress curves were superimposable. This observation indicates that DnaK is saturated with GrpE under these conditions, thus binding must be very tight: we estimate an upper limit for K_d of 50 nM. The lack of any observable dissociation of the GrpE.DnaK complex during SEC HPLC (see below) also suggests a nanomolar or lower K_d.

Oligomeric state of GrpE

Analysis by SEC HPLC (see Fig. 1) showed native GrpE to elute as a single peak corresponding to molecular mass of 193 kDa, although its subunit molecular mass is 23 kDa. This elution volume would correspond to an octamer of GrpE, if it were globular. Under denaturing PAGE conditions GrpE migrates with a molecular mass of 23 kDa. Using native PAGE, GrpE has a mobility almost identical to that of DnaK corresponding to a molecular mass of 67 kDa. However, because migration of proteins in native gels is dependent on charge and shape, an alternative method was sought to determine the oligomeric state of native GrpE. GrpE was cross-linked with glutaraldehyde under native conditions and then subjected to SDS-PAGE. The gel bands were broad as a result of the cross-linking, and the cross-linked GrpE migrated to a position corresponding to a MW of 55 ± 5 kDa, suggesting either a dimer or trimer. From the SEC HPLC data we calculate a

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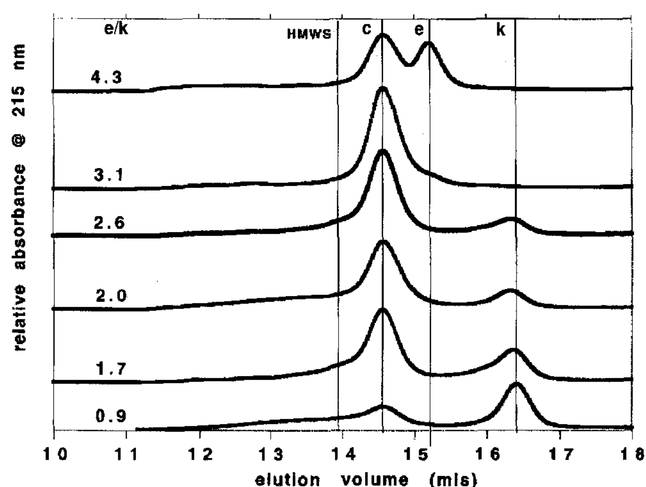

Fig. 1 Titration of DnaK by GrpE followed at 215 nm by SEC HPLC. The vertical lines indicate the positions of high molecular weight species (HMWS); GrpE.DnaK complex (c); GrpE (e); and DnaK (k). The numbers on the left side of the chromatogram indicate the monomer ratio of GrpE to DnaK. Protein concentrations used for GrpE:DnaK ratio were: (0.9) 9.1 μM DnaK and 7.8 μM GrpE; (1.7) 9.1 μM DnaK and 15.5 μM GrpE; (2.0) 9.1 μM DnaK and 18.6 μM GrpE; (2.6) 9.1 μM DnaK and 23.3 μM GrpE; (3.1) 9.1 μM DnaK and 27.9 μM; (4.3) 9.1 μM DnaK and 38.8 μM GrpE.

Stokes radius (R_s) for native GrpE of 47.2 ± 1 Å. The calculated R_s for monomeric GrpE, if it were spherical is 27.0 Å. Clearly, GrpE has a highly asymmetric shape, e.g. for a prolate ellipsoid we estimate the axial ratio would be approximately 5.3.

Stoichiometry of the DnaK.GrpE complex

A mixture containing GrpE and DnaK was incubated for 10 min at 37°C to form the complex, and was subsequently isolated by SEC HPLC. The complex was well resolved from free GrpE and free DnaK, as can be seen in Figure 1. The fraction containing the complex was run on an SDS 8–25% acrylamide gel, along with the following known ratios of GrpE to DnaK: 0.5:1, 1:1, 3:1 and 2:1. Densitometer traces of the SDS gel were used to determine the area of GrpE and DnaK in each lane of the gel. The relative response factor of DnaK and GrpE to Coomassie blue R staining was determined using known concentrations of DnaK and GrpE. In order to minimize the error, the sample collected from the HPLC was run in two separate lanes and each lane was scanned four times. The average value for the response factor, k, was determined as 6.15 ± 0.45. Knowing the response factor (k) and the areas of DnaK and GrpE in the HPLC fraction, determined from densitometric tracings, the stoichiometry of the GrpE.DnaK complex can be calculated. The experiment was repeated using an excess of DnaK over GrpE to form complex on the HPLC. This was done to ensure that all the GrpE would be in the complex, and therefore that there was no possibility of free GrpE in the stoichiometry determination. In both sets of experiments the ratio of GrpE to DnaK in the complex was determined to be 3.5 ± 0.50.

The stoichiometry was also investigated using gel filtration HPLC analysis. Increasing amounts of GrpE were added to DnaK until only the complex peak was evident. Figure 1 shows that, when GrpE is added at a 3-fold molar excess to DnaK, only the complex peak is evident. Adding more than a 3-fold molar excess of GrpE results in a new peak representing free GrpE, and no free DnaK peak is evident. Also, high molecular weight species (HMWS) of DnaK are seen when GrpE is present at less than a 3-fold excess over DnaK. With constant DnaK concentration, there is a decrease in the amount of HMWS with increasing concentrations of GrpE, suggesting that GrpE facilitates the dissociation of the oligomers to the monomeric form of DnaK, through preferential binding to the monomeric form.

Effect of nucleotides on the DnaK.GrpE interaction

Equilibrium dialysis experiments showed that GrpE has no affinity for either ATP or ADP. The kinetics of GrpE.DnaK complex formation were monitored by SEC HPLC and fluorescence. As noted, with the Bio-SEP SEC HPLC column, GrpE elutes as a single peak corresponding to molecular mass of 193 kDa, although its subunit MW is 23 kDa. DnaK elutes more than a minute later than GrpE, corresponding to an apparent molecular mass of 89 kDa (DnaK, molecular mass 69 kDa, is also a significantly asymmetric molecule). The GrpE.DnaK complex elutes at 14.5 min, separated from free GrpE by 0.7 min, corresponding to an apparent molecular mass of 260 kDa.

The amount of complex formed by mixing GrpE and DnaK was studied as a function of increasing incubation time at 37°C using 16 μM DnaK and 12.8 μM GrpE. The maximum amount of complex (i.e. no free GrpE) was formed at the earliest time of sampling. Similar results were obtained in the presence of 0.2 mM ADP in the incubation mixture and 50 μM ADP in the running buffer. However, in the presence of 0.2 mM ATP in incubation mixture and 50 μM ATP in the running buffer no complex was detected either initially or with increasing incubation time. Thus, in the presence of ATP the complex either does not form, or is unstable and dissociates very rapidly. If ATP was not present in the HPLC running buffer but present only in the incubation mixture, the maximum amount of complex was observed, indicating that the complex can reform during the initial HPLC separation, as ATP was retarded from the proteins in the HPLC column.

The rapid formation of the GrpE.DnaK complex was

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confirmed in fluorescence kinetics experiments, which showed the formation of complex to be complete within the dead-time of manual mixing, i.e. no time-dependent transients were observed, and the emission spectrum taken at the earliest possible time showed that the blue shift corresponding to the presence of the GrpE.DnaK complex (see below) had already occurred. Based on these experiments, we estimate the rate of complex formation to be $k_{on} > 1\ s^{-1}$, whereas the off-rate is very slow on the HPLC timescale ($k_{off} \leq 10^{-4}\ s^{-1}$).

Conformation of the Dnak.GrpE complex

The effects of GrpE on the conformation of DnaK in the absence and presence of nucleotides was investigated using fluorescence and circular dichroism. DnaK has a single tryptophan residue in position 102 (Bardwell and Craig 1984) located in the ATP-binding domain of the protein. GrpE has no tryptophans and one tyrosine and, therefore, has minimal fluorescence signal when excited at 280 nm. Figure 2 shows the fluorescence emission of DnaK at 22°C in the presence of GrpE and ATP. ATP causes a small blue shift in the λ_{max} as previously observed (Palleros et al 1992). GrpE induces the same blue shift on DnaK as seen with ATP, and no further change is seen in the presence of both GrpE and ATP. In fact, the same blue shift is seen in the fluorescence spectrum of DnaK when in the presence of GrpE, ATP, GrpE + ATP and GrpE + ADP. In contrast, reduced, carboxymethylated ribonuclease A (RC-RNase), an unfolded substrate for DnaK which has no Trp residues, had no effect on the emission spectra of DnaK (data not shown). In addition to the blue shift in λ_{max}, binding of GrpE to DnaK also results in some fluorescence quenching, although not as much as seen with binding of ATP and ADP.

The far UV-CD spectrum of GrpE indicates a substantial amount of α-helix, and closely resembles the CD spectrum of DnaK (Fig. 3). To determine whether the interaction of GrpE and DnaK resulted in a conformational change we collected spectra of the individual proteins as well as their mixture. By subtracting the spectrum of GrpE alone from the mixture with DnaK, we obtained a difference spectrum. As shown in Figure 3, the addition of GrpE to DnaK results in no change in the CD spectrum, indicating the absence of any significant change in the secondary structural conformation on formation of the DnaK.GrpE complex. Within experimental error the observed CD spectrum for the complex is the sum of that of DnaK and GrpE alone. In addition, there is no change in the secondary structure of the DnaK.GrpE complex in the presence of ATP or ADP (Fig. 3), or unfolded substrate, reduced carboxymethylated α-lactalbumin (RCMLA) (data not shown).

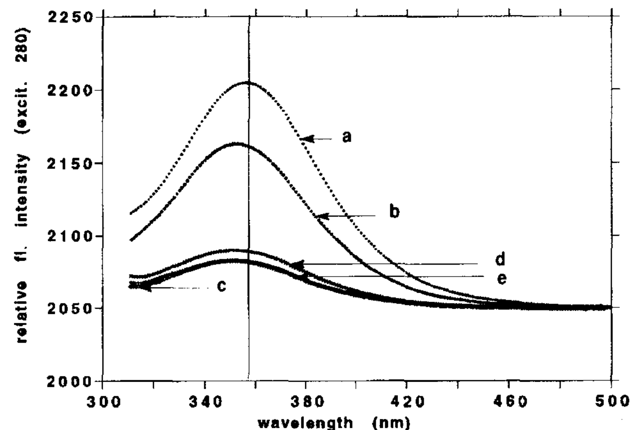

Fig. 2 Interaction between DnaK and GrpE followed by fluorescence. a, DnaK; b, DnaK and GrpE; c, DnaK and 537 μM ATP/Mg; d, DnaK, GrpE and 537 μM ATP/Mg; e, DnaK, GrpE and 537 μM ADP/Mg. Excitation was at 280 nm. Excitation slit: 3 nm; emission slit: 5 nm. Protein concentrations: 2.0 μM DnaK and 3.7 μM GrpE. All spectra were collected at 21.5°C in 20 mM Tris, 100 mM KCl, pH 7.1. The spectrum of GrpE was subtracted from all spectra containing both DnaK and GrpE. The fluorescence spectra are uncorrected.

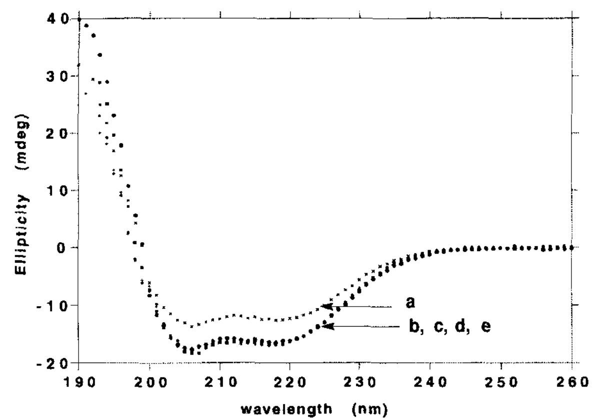

Fig. 3 Interaction between DnaK and GrpE followed by far UV-CD. a, GrpE; b, DnaK; c, the mixture of DnaK and GrpE minus the spectrum of GrpE; d, the mixture of DnaK, GrpE and 200 μM ATP/Mg minus the spectrum of GrpE; e, the mixture of DnaK, GrpE and 200 μM ADP/Mg minus the spectrum of GrpE. All spectra were collected at 37°C in 20 mM Tris, 100 mM KCl, pH 7.1. Note that the spectrum of GrpE was subtracted from all spectra containing both DnaK and GrpE. Protein concentrations: 5.9 μM DnaK and 16.2 μM GrpE.

Thermal stability monitored by far UV-CD

To further investigate the nature of the interaction between GrpE and DnaK we examined the thermal denaturation of the GrpE.DnaK complex. The thermal denaturation of DnaK involves two unfolding transitions, the first transition is more cooperative (T_m = 42°C) with 25% loss of secondary structure leading to a partially-folded intermediate, the second, a much less cooperative

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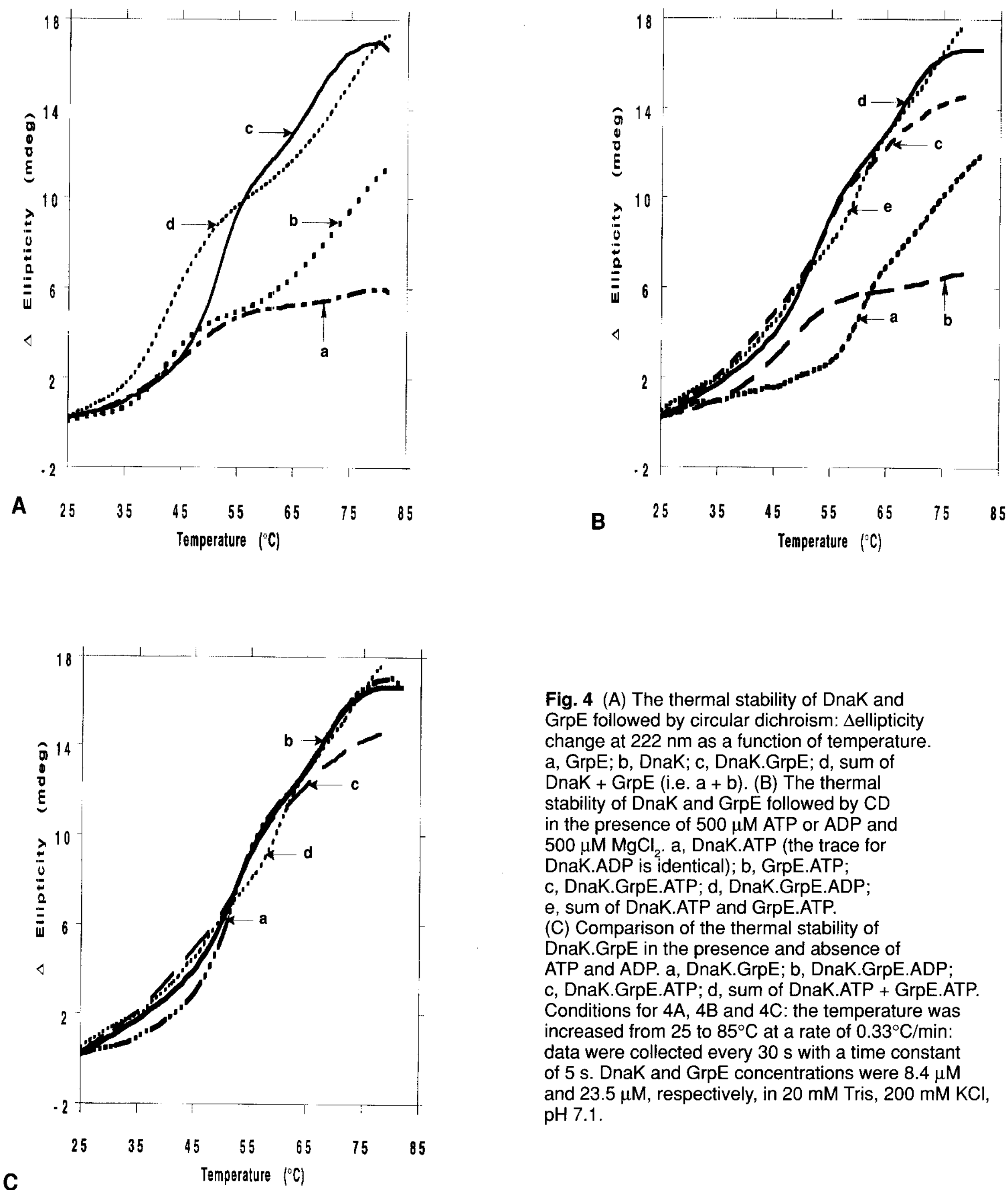

Fig. 4 (A) The thermal stability of DnaK and GrpE followed by circular dichroism: Δellipticity change at 222 nm as a function of temperature. a, GrpE; b, DnaK; c, DnaK.GrpE; d, sum of DnaK + GrpE (i.e. a + b). (B) The thermal stability of DnaK and GrpE followed by CD in the presence of 500 μM ATP or ADP and 500 μM $MgCl_2$. a, DnaK.ATP (the trace for DnaK.ADP is identical); b, GrpE.ATP; c, DnaK.GrpE.ATP; d, DnaK.GrpE.ADP; e, sum of DnaK.ATP and GrpE.ATP. (C) Comparison of the thermal stability of DnaK.GrpE in the presence and absence of ATP and ADP. a, DnaK.GrpE; b, DnaK.GrpE.ADP; c, DnaK.GrpE.ATP; d, sum of DnaK.ATP + GrpE.ATP. Conditions for 4A, 4B and 4C: the temperature was increased from 25 to 85°C at a rate of 0.33°C/min: data were collected every 30 s with a time constant of 5 s. DnaK and GrpE concentrations were 8.4 μM and 23.5 μM, respectively, in 20 mM Tris, 200 mM KCl, pH 7.1.

transition*. spans a large temperature range from 60 to 90°C (Fig. 4A) (Palleros et al 1992). In contrast, GrpE undergoes a single cooperative unfolding transition, with a T_m = 43°C, a very similar value to that of the first unfolding transition of DnaK (Fig. 4A). The presence of ATP or ADP (0.35 mM) had no effect on the thermal stability of GrpE (Fig. 4B, Table 1). These results are in accord with our finding that GrpE has no affinity for either ATP or ADP, as determined by equilibrium dialysis experiments (unpublished results).

* The second transition actually corresponds to two overlapp-ing transitions, which are not well-resolved by circular dichroism or fluorescence (Montgomery et al 1993; Shi et al 1994).

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Table 1 Thermostability of GrpE, DnaK and their complexes

Components	Midpoint[a] of the first transition (°C)
DnaK	42
GrpE	43
DnaK + ATP	59
GrpE + DnaK	51
GrpE + ATP	44
GrpE + DnaK + ATP	50
GrpE + DnaK + ADP	52

Conditions were 20 mM Tris, 200 mM KCl, pH 7.1, 8.4 μM DnaK, 23.5 μM GrpE, 500 mM nucleotide.
a Estimated error is ± 1°C.

The thermal unfolding of a mixture of DnaK (8.4 μM) and GrpE (23.5 μM), which forms a stable complex at 37°C, exhibits multiple unfolding transitions. The first transition, with T_m = 51°C, is 9°C higher than that of either DnaK or GrpE alone and more cooperative (Fig. 4A), consistent with the expected greater stability of the complex. The second unfolding transition has a midpoint at 67°C.

Both ATP and ADP significantly increase the thermal stability of DnaK in that the midpoint of the first transition is shifted to 59°C (Palleros et al 1992). The thermal unfolding transition of a mixture of DnaK, GrpE and ATP is different from that of the GrpE.DnaK complex and similar to the sum of the individual components in the presence of ATP, i.e. GrpE.ATP + DnaK.ATP, except that at higher temperatures it is shifted a few degrees lower than that for DnaK.ATP (Fig. 4B, C). In the presence of ADP, the midpoint of unfolding transition of the mixture of DnaK and GrpE was similar to that seen in the absence of ADP; however, the first transition is less cooperative (Fig 4C). Table 1 summarizes the midpoints of the first thermal transitions for DnaK and GrpE and their complexes.

To determine whether the initial transition of the GrpE.DnaK mixture, with T_m = 51°C, corresponded to dissociation of the GrpE.DnaK complex, we investigated the events occurring during the thermal unfolding in the temperature range of 50–65°C using SEC HPLC. DnaK forms a molten globule or partially-folded intermediate when incubated at 50°C (Palleros et al 1992). This intermediate state can be distinguished from the native state by SEC HPLC, and was shown not to bind the unfolded substrate RCMLA. Figure 5A shows that when DnaK (9.1 μM) and GrpE (27.9 μM) were incubated at 65°C for 10 min and injected directly into the HPLC at 23°C, at least 93% of the mixture was in the form of the complex, which is inconsistent with the hypothesis that the initial transition corresponded to dissociation of the GrpE.DnaK complex. If the DnaK molten globule state is

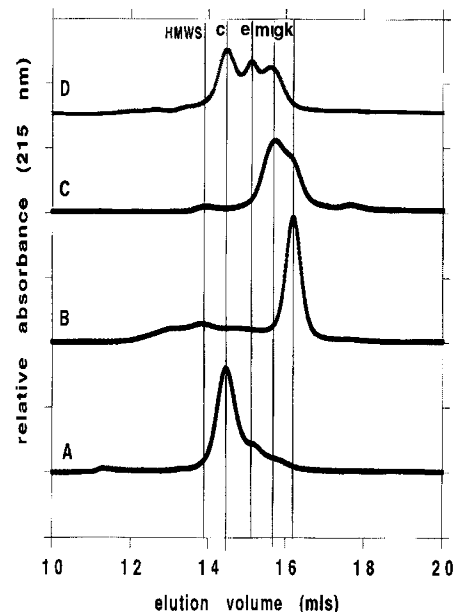

Fig. 5 HPLC analysis of DnaK.GrpE interaction at high temperatures; k = DnaK, e = GrpE, C = GrpE.DnaK complex, mg = molten globule. a, DnaK and GrpE incubated at 65°C for 10 min; b, DnaK (no incubation); c, DnaK incubated at 55°C for 10 min; d, DnaK incubated at 55°C for 10 min, cooled to 22°C, and GrpE then added. All samples were injected immediately after incubation. Protein concentrations: DnaK (9.1 μM) and GrpE (27.9 μM) in 20 mM Tris, pH 7.1.

preformed (50°C for 10 min), stabilized by cooling to 23°C and GrpE is then added and the mixture immediately injected into the HPLC, much less complex is evident (34%) (Fig. 5D). Furthermore, there is no evidence of native DnaK (Fig. 5B), only a peak corresponding to molten globule, indicating that the complex formed from the small amount of native DnaK present under these conditions. Refolding of the molten globule in the presence of GrpE was examined as a function of time: the kinetics of formation of complex were similar to those for the formation of native DnaK in the absence of GrpE. For example, after incubation of the molten globule (9.1 μM) with GrpE (27.9 μM) at 20°C for 2 h, 62% complex was present (data not shown).

DISCUSSION

The GrpE.DnaK complex

GrpE forms a very tight complex with DnaK. The ATPase data indicate a maximum K_d of 50 nM. The SEC HPLC data in Figure 1 show that the off-rate is negligible on the HPLC timescale ($k_{off} \leq 10^{-4}$ s^{-1}) which, given a reasonable estimate for a diffusion-controlled bimolecular binding constant, suggests a nM or lower dissociation constant.

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Binding of GrpE to DnaK is fast, based on the observation that the blue shift in the λ_{max} of DnaK on binding GrpE was complete within the few seconds required for manual mixing, and that the maximum amount of complex was seen by SEC HPLC immediately after mixing GrpE and DnaK. These observations are in contrast to the report by Schonfeld et al (1995) who showed maximum complex formation after 15 h. We believe this discrepancy arises from the fact that in their experiment the rate-limiting step was dissociation of oligomeric DnaK (some of which may have been stabilized by bound ADP resulting from incomplete removal of nucleotides during purification).

It was originally reported that GrpE binds to DnaK in a 1:1 ratio (Zylicz et al 1987), and more recently as a 2:1 ratio (Schonfeld et al 1995). The error in the original estimate of a 1:1 ratio was attributed to the fact that sedimentation data were analyzed assuming a globular shape for GrpE (Schonfeld et al 1995). Our data also indicate that GrpE is not globular in shape, based on the elution volume in SEC HPLC, which corresponds to a molecular mass of 193 kDa. The extreme asymmetric shape complicates determination of the quaternary structure of GrpE under native conditions. Both the native PAGE and cross-linking experiments suggest GrpE exists as a dimer or trimer. However, both of these techniques could be skewed by the elongated shape of GrpE. Recently, Schonfeld et al (1995) used sedimentation equilibrium ultracentrifugation to investigate the quaternary state of GrpE and concluded it exists as a dimer under native conditions.

Since GrpE and DnaK form a stable complex, it was possible to perform a titration of DnaK with GrpE to determine the stoichiometry of the complex using SEC HPLC. A single peak is observed at a molar ratio of 3:1 of GrpE to DnaK, and an excess of either DnaK or GrpE is well resolved from the complex peak (Fig. 1). This stoichiometry is in agreement with that obtained by SDS-PAGE analysis of the GrpE.DnaK complex isolated by SEC HPLC. Since both experiments are dependent on accurate determination of the concentration of GrpE and DnaK, the molar extinction coefficient at 280 nm for both DnaK and GrpE was determined using three different methods. All three methods were in close agreement, and are in accord with previously reported values (Hartl et al 1992). Due to its small extinction coefficient at 280 nm, 1500 M^{-1} cm^{-1}, GrpE in the 1–40 μM range was undetectable by HPLC when followed at 280 nm at the lowest range setting of 0.02 AFS. This observation is in contrast with previously reported gel filtration analysis, in which a rather substantial peak was detected for uncomplexed GrpE (in this concentration range) monitored at 278 nm (Schonfeld et al 1995). Considering that there are no tryptophans, and only one tyrosine in GrpE, one would not expect a significant absorbance at 278 nm.

We are unable to account for the apparent discrepancy that native GrpE appears to be a dimer, and yet in complex with DnaK the stoichiometry appears to be 3:1 GrpE to DnaK. Clearly, the stoichiometry experiments are critically dependent on accurate knowledge of the concentrations of the two proteins; however, there is no reason to suspect that our values of the extinction coefficient at 280 nm, used in these determinations, are wrong. The ATPase data indicate that both DnaK and GrpE are fully catalytically active. Our data are also inconsistent with the complex containing more than one molecule of DnaK. It is most reasonable to assume that either native GrpE is a dimer and forms a complex with DnaK containing two molecules of GrpE, or that native GrpE is a trimer and forms a complex with DnaK containing three molecules of GrpE to one of DnaK. At this point it is unclear which of these is correct; however, given the marked asymmetry of GrpE it is perhaps more likely that the stoichiometry data are correct and that native GrpE is actually a trimer.

In agreement with previous studies (Schonfeld et al 1995), we find that increasing amounts of GrpE decrease the amount of DnaK oligomers, indicating that GrpE binds to the monomeric form of DnaK. We have previously shown that the monomer of DnaK is the active species in ATP hydrolysis and unfolded substrate protein binding (Palleros et al 1993b).

Effect of GrpE on thermal stability of DnaK

One way in which to ascertain the strength of the interaction between two proteins is to examine their stability, if the interaction is strong then the stability of the complex will be greater than that of the individual components. As indicated in Figure 4 and Table 1, the formation of a complex between DnaK and GrpE results in an increase in the thermal stability of about 9°C in the first transition midpoint, 51°C, compared to those of the individual proteins, DnaK 42°C, and GrpE 43°C. The complex also results in a second, less cooperative, unfolding transition with a midpoint of 67°C. Our interpretation is that the complex of GrpE and DnaK undergoes an initial thermal transition (T_m 51°C), resulting from a conformational change of the complex due to the *partial* unfolding of one or both of the proteins. The second transition with T_m 67°C corresponds to the dissociation of the complex and unfolding of both components. The HPLC data show that at 65°C, well above the first transition, nearly 100% of complex is present (Fig. 5). We have previously shown that DnaK and RCMLA do not form complex above 50°C despite the similar T_m values seen for the unfolding transitions of DnaK.GrpE and DnaK.RCMLA. To rule out the possibility that GrpE facilitates refolding, thus allowing

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the rapid association of the complex, we showed that GrpE does not bind to the molten globule and that the recovery of the molten globule to the native state in the presence of GrpE is slow; $t_{1/2} > 1$ h at 20°C. Analysis of a mixture of GrpE and DnaK incubated at 55°C indicated that only 34% of the potential complex was present and that all the free DnaK was in the molten globule state. This indicates that the GrpE binds to the native state of DnaK but not the partially-folded intermediate state. As refolding of the molten globule to native state continued with time, a further increase in the amount of complex was observed (data not shown). If the first unfolding transition represented the dissociation of the complex, at 65°C we would expect to see DnaK in the molten globule state and complex formation would be a slow process; instead we see nearly all complex formed at 'time zero'. The stoichiometry of the complex is also maintained at high temperature (65°C) indicated by the fact that nearly 100% complex is seen and an excess of GrpE is not evident. Therefore, the first unfolding transition does not represent the dissociation of the GrpE.DnaK complex. The lack of effect of ATP or ADP on the thermal stability of GrpE is consistent with our equilibrium dialysis experiments indicating that the cofactor does not interact with these nucleotides.

The thermal transition for the mixture of GrpE, DnaK and ATP suggests that the presence of ATP weakens the complex, leading to dissociation and a mixture of GrpE and the binary DnaK.ATP complex. However, the fact that the second major transition in this case is at a lower temperature than found for DnaK.ATP in the absence of GrpE suggests that GrpE transiently interacts with this binary complex to dissociate the ATP, leading to some free DnaK and the apparently lower transition temperature, 51°C, for the mixture compared to 59°C for DnaK.ATP. This interpretation is consistent with the role of GrpE as a nucleotide exchange factor (increasing the rate of nucleotide dissociation), and the observation that in the presence of ATP no DnaK.GrpE complex is observed by SEC HPLC. The presence of ADP had little effect on the thermal transition of the GrpE.DnaK complex, consistent with the SEC HPLC results indicating no effect of ADP on the complex.

Does binding of GrpE to DnaK induce a conformational change?

In order to investigate the physical interaction between DnaK and GrpE, we examined the conformations of DnaK induced by GrpE in the absence and presence of ATP and ADP. We have previously shown that ATP induces a conformational change in DnaK which is manifested by a blue shift in the λ_{max} of the tryptophan fluorescence, but no changes in the circular dichroism spectrum (Palleros et al 1992). Binding of GrpE to DnaK has similar effects, namely no change in the circular dichroism spectrum, indicating no change in the secondary structure of either protein on formation of the complex, and a similar blue shift in the λ_{max} of DnaK to that brought about by ATP, indicating a change in the tertiary structure.

The fact that the blue shift is also evident in the presence of ADP suggests that ADP does not alter the interaction between DnaK and GrpE and that the change is due to the binding of GrpE, since ADP alone does not induce a blue shift in DnaK (Palleros et al 1992). However, the similar shift in λ_{max} for DnaK in the presence of ATP and GrpE is most likely due to the effects of ATP on DnaK, since our data indicate that ATP dissociates the GrpE.DnaK complex, as previously suggested (Zylicz et al 1987). In contrast to binding of GrpE, binding of unfolded substrates, such as RCMLA and RC-RNase, resulted in no change in the fluorescence of DnaK. These observations reflect that substrates and regulators mediate DnaK action by inducing different conformations in the protein. Our data do not exclude the possibility that unfolded substrate causes a conformational change in the C-terminal domain which does not change the environment of the tryptophan located in the ATPase domain.

Models for GrpE-catalyzed nucleotide exchange

Our model for the DnaK reaction cycle in the absence of cofactors (Palleros et al 1993a) proposes that binding of ATP to DnaK, or DnaK-substrate protein complexes, results in a conformational change in DnaK such that it has low affinity for the substrate protein (due to rapid off-rates for the substrate). The most likely role of DnaJ is to facilitate binding of unfolded substrate proteins to the DnaK.ATP complex, through initial formation of a DnaJ.U complex. DnaJ has been shown to bind significantly only to DnaK when DnaK is complexed to ATP. Binding of DnaK.ATP to DnaJ.U leads to formation of a complex of DnaK.U.ADP. GrpE catalyzes the exchange of ATP for ADP in this complex, leading to a conformational change resulting in the low-affinity form of DnaK, K*, and dissociation of the substrate protein.

The simplest model for how GrpE facilitates nucleotide exchange is based on the fact that both ATP and GrpE induce similar shifts in the λ_{max} for Trp fluorescence in DnaK. Assuming that both ATP and GrpE induce the same conformation of DnaK, K*, which has low affinity for the substrate protein and preferential binding of ATP, and that ADP and unfolded substrate protein or peptides shift the equilibrium in favor of K, the high affinity conformation, then the nucleotide exchange properties of GrpE would be explained by the fact that GrpE shifts the

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conformational equilibrium of DnaK to the conformation K*, which has low affinity for ADP and high affinity for ATP. However, there are two problems with this simple model: it predicts that GrpE, in the absence of ATP, should favor release of the unfolded substrate protein from DnaK, but this does not occur (e.g. adding GrpE to the DnaK.RCMLA complex results in a stable ternary complex which can be isolated by SEC HPLC (data not shown)), and this model does not account for the dissociation of the GrpE.DnaK complex in the presence of ATP. Thus, it is likely that the conformation changes induced in DnaK by GrpE and by ATP are different, even though they both lead to the same effect on λ_{max} for fluorescence emission.

An alternative model is one in which binding of GrpE to DnaK leads to a tertiary structural change involving the ATP-binding domain (which contains the single Trp residue). It has previously been shown that the highly conserved loop in the ATPase domain in DnaK is necessary for binding to GrpE (Buchberger et al 1994). Based on our fluorescence results, one can speculate that binding of GrpE alters the loop structure leading to opening of the cleft responsible for nucleotide release (Buchberger et al 1994). The tertiary structural change could be limited to only the loop region or may represent a more global change in the ATPase domain. The fact that there is no change in the far UV-CD spectrum of DnaK in the presence of GrpE, ATP or ADP indicates that the conformational change brought about by GrpE is manifested in tertiary structure changes. Given that the ATP-binding domain of Hsp70s is structurally homologous to hexokinase (Flaherty et al 1990), it is likely that the conformational change induced by GrpE is analogous to that induced in hexokinase by its glucose substrate, involving rotations of whole domains.

The possibility that GrpE may elicit some of the same functions in DnaK as ATP is supported by the observation that high levels of Mg^{2+} eliminate the requirement for GrpE in the monomerization of RepA in plasmid P1 replication and, conversely, low levels of Mg^{2+} increase the requirement for GrpE (Skowyra and Wickner 1993). Since it is the Mg^{2+} salt of ATP which is essential for the activity of ATP with DnaK, it is likely that low levels of Mg^{2+} result in significantly diminished binding of ATP to DnaK.

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