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# The DnaK Chaperone System of *Escherichia coli*: Quaternary Structures and Interactions of the DnaK and GrpE Components*

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**The DnaK (Hsp70), DnaJ, and GrpE heat shock proteins of *Escherichia coli* constitute a cellular chaperone system for protein folding. Substrate interactions are controlled by the ATPase activity of DnaK which itself is regulated by the nucleotide exchange factor GrpE. To understand the structure-function relationship of this chaperone system, the quaternary structures of DnaK, GrpE, and DnaK-GrpE complexes were analyzed by gel filtration chromatography, dynamic light scattering, analytical ultracentrifugation, and native gel electrophoresis. GrpE formed dimers in solution. DnaK formed monomers, dimers, and higher mole mass oligomers, the equilibrium between these forms being dependent on the DnaK concentration. The behavior of DnaK and GrpE in gel filtration and dynamic light scattering suggested elongated shapes of both molecules. In the absence of added nucleotides, DnaK and GrpE formed stable complexes containing one molecule of DnaK and two molecules of GrpE. A 44-kDa N-terminal ATPase fragment of DnaK also formed complexes with GrpE with the same 1:2 stoichiometry. DnaK-GrpE complex formation was unaffected by elimination of DnaK-bound nucleotides or addition of saturating concentrations of a DnaK peptide substrate. These findings allow the correlation of DnaK-GrpE interactions with a role for GrpE in the functional cycle of the DnaK chaperone system.**

Hsp70 stress proteins (Hsp70) are molecular chaperones that assist folding and degradation of proteins as well as disassembly of protein complexes (1). A key feature of their chaperone function is the ability of Hsp70 to bind nonnative protein substrates and to release them upon ATP binding (2).[1] The ATPase activity of at least some Hsp70 proteins is under tight control of cofactors allowing coordination of ATP binding/hydrolysis and substrate interaction. For the *Escherichia coli* homologue DnaK, its low intrinsic ATPase activity is jointly stimulated by the DnaJ and GrpE heat shock proteins acting at different steps of the reaction (3). In particular, GrpE is a nucleotide exchange factor of DnaK (3, 4) and, apparently as a consequence of this activity, mediates the efficient release of DnaK-bound substrates thereby allowing recycling of DnaK (5). GrpE is essential for chaperone activities of DnaK *in vivo* (4) and *in vitro* (5, 6).

Limited information exists on the quaternary structures of DnaK, DnaJ, and GrpE, and the complexes formed between them (1). DnaK was found to form monomers and a minor fraction of dimers in gel filtration chromatography and monomers in glycerol gradient sedimentation (7). GrpE has a relative mole mass (mole mass) of 22 kDa and was suggested to form monomers based on glycerol gradient sedimentation analysis (8). DnaK and GrpE interact both *in vivo* and *in vitro* (9). DnaK-GrpE complexes are stable in the absence of added nucleotides and are disrupted by $Mg^{2+}$/ATP (8). A loop structure exposed at the surface of the N-terminal ATPase domain of DnaK is essential for stable GrpE binding (10).

To elucidate the relation of structure and function of the DnaK system we analyzed in detail the quaternary structures of DnaK, GrpE, and DnaK-GrpE complexes with a variety of biochemical and biophysical methods. The hydrodynamic parameters determined in the present study offer new insight into the quaternary structures of these proteins and the role of DnaK-GrpE interactions in the functional cycle of the DnaK chaperone system.

## MATERIALS AND METHODS

*Protein Purification*—DnaK and GrpE were overproduced in *E. coli* cells (strain W3110) carrying pDS56/RBSII (*dnaK*) or pDS56/RBSII (*grpE*) upon isopropyl-1-thio-β-D-galactopyranoside-induction of expression of the plasmid-encoded chaperone genes (11). The chaperones were purified by ammonium sulfate fractionation (only GrpE), DEAE-Sepharose chromatography, ATP-agarose chromatography (only DnaK), hydroxylapatite chromatography, and Sephacryl S-300 gel filtration according to published procedures (7, 8, 12) with modifications[2] and stored at −80 °C until usage. The DnaK and GrpE preparations are homogeneous as determined by amino acid analysis, mass spectroscopy and N-terminal sequencing and more than 98% pure as determined by densitometric analysis of the proteins subjected to SDS-PAGE[3] (Fig. 1*A*).

The 44-kDa ATPase fragment of DnaK (DnaK1–385) was overproduced in *E. coli* cells (strain MC4100) carrying pUHE21-2fdΔ12 (*dnaK1–385*) (13) upon isopropyl-1-thio-β-D-galactopyranoside induction of expression of the plasmid-encoded gene and was purified as described (10).

Preparation of nucleotide-free DnaK was done by J. Reinstein and will be described elsewhere.[4] Protein concentrations were determined by colorimetric assays (BCA protein assay; Pierce) and calibrated by amino acid analysis.

Purified DnaK and GrpE were fully active by several criteria, including chaperone-mediated refolding of firefly luciferase (DnaK and GrpE), ATPase activity (DnaK and DnaK1–385), and substrate binding (DnaK) (6, 13).

*Analytical Gel Filtration Chromatography*—In standard gel filtration experiments, aliquots of 50 μl of protein samples were loaded onto

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[1] J. McCarty, A. Buchberger, J. Reinstein, and B. Bukau, submitted for publication.

[2] H.-J. Schönfeld *et al.*, manuscript in preparation.

[3] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; QLS, quasi-elastic light scattering.

[4] J. Reinstein *et al.*, manuscript in preparation.

a Superose 12 column (1 × 30 cm; Pharmacia LKB) equilibrated with 50 mM Tris-HCl, pH 7.7, 100 mM NaCl at room temperature and were chromatographed at a flow rate of 0.5 ml/min. For experiments with nucleotide-free DnaK or with peptide C, a Superdex 200 column (1 × 30 cm; Pharmacia LKB) equilibrated with 25 mM HEPES-HCl, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5 mM EDTA, 10% glycerol was used. Fast gel filtration was performed at 1 ml/min where indicated. Elution profiles where detected at 278 nm. Gel filtration standards were from Bio-Rad.

*Laser Light Scattering*—Samples were filtered through 0.2-μm pore-sized Nucleopore membranes (Nucleopore, Cambridge, MA) into the cylindrical measuring cell placed in a temperature-controlled toluene bath (20 °C) and examined with the ALV-3000 laser-scattering system (ALV, Langen, Federal Republic of Germany). Correlation functions were recorded at a scattering angle of 90 ° and analyzed with the program CONTIN (14). When the distribution of diffusion coefficients was unimodal and narrow, the mean value (quoted in Table I) was calculated as the second moment of the distribution. When it was unimodal and broad, the first and third moment was taken to indicate the range of compatible values of the diffusion coefficient. When more than one peak was present, each peak was quantified by its second moment. The diffusion coefficient D is related to the hydrodynamic radius R by the Stokes-Einstein relation,

$$D = kT/(6\pi\eta R) \quad \text{(Eq. 1)}$$

where $k$ is the Boltzmann constant, $T$ the temperature, and $\eta$ the viscosity of the solvent ($\eta$ = 1.017 mPa s at 20 °C). For a globular protein of mass $M$ the radius $R$ can be estimated from the following equation,

$$4\pi R^3/3 = M\bar{v}h/N_{av} \quad \text{(Eq. 2)}$$

where $\bar{v}$ is the partial specific volume ($\bar{v}$ = 0.735 $cm^3/g$), $h$ the hydration of typical proteins ($h$ = 1.3), and $N_{av}$ Avogadro's number.

*Analytical Ultracentrifugation*—Measurements were made with the analytical ultracentrifuge Optima XL-A (Beckman, Palo Alto, CA). Sedimentation velocity runs were carried out at 40,000 rpm and 20 °C using a 12-mm double sector cell. Samples (about 350 μl) were analyzed at 1 mg/ml (DnaK), 0.6 mg/ml (GrpE), and 1.6 mg/ml (DnaK-GrpE mixture), respectively. Sedimentation equilibrium runs were done at 7,000 rpm and 20 °C with sample volumes of about 100 μl. Equilibrium profiles recorded at 280 nm were analyzed as published (15).

From the observed diffusion coefficients D obtained in light scattering and the sedimentation velocities $s$ the mole masses were calculated using the following Svedberg equation,

$$M = s/D \times RT/(1 - \bar{v}\rho) \quad \text{(Eq. 3)}$$

where $R$ is the gas constant and $\rho$ the density of the buffer ($\rho$ = 0.999 $g/cm^3$).

*Polyacrylamide Gel Electrophoresis*—Denaturing SDS-PAGE according to the method of Laemmli (16) was performed under reducing conditions using precast gels (16% Tris-glycine gel; Novex, San Diego, CA) running at 150 V for 1.5 h. Gels were stained with Serva Blue R (Serva, Heidelberg, Federal Republic of Germany). Protein standards were from Novex (Mark 12). Quantification of stained bands was performed using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Nondenaturing PAGE (8% acrylamide) was performed as described (10), and the relative amounts of GrpE, DnaK, and GrpE-DnaK complexes were determined using a Hirschmann Elscript 400 scanner.

## RESULTS

*Quaternary Structure of GrpE*—The quaternary structures of GrpE, DnaK, and DnaK-GrpE complexes were analyzed by gel filtration, dynamic light scattering, and analytical ultracentrifugation. In gel filtration experiments, GrpE eluted as a single symmetrical peak after injection into a Superose 12 column (Fig. 1*B*). The elution volume of the peak maximum corresponds to an apparent mole mass of 190 kDa, relative to globular reference proteins. When GrpE was analyzed by gel filtration in the presence of 1 M NaCl, the position of the eluted peak was not significantly changed relative to reference proteins (not shown).

In quasi-elastic light scattering (QLS) experiments, the correlation functions obtained indicate a slightly broader size distribution of GrpE than expected for a strictly monodisperse sample of globular protein. When the data were fitted to a single exponential, diffusion coefficients of 4.8–5.6 × $10^{-7}$ $cm^2$ $s^{-1}$ were obtained for different preparations of GrpE at a concentration of 0.6 mg/ml (Table I).

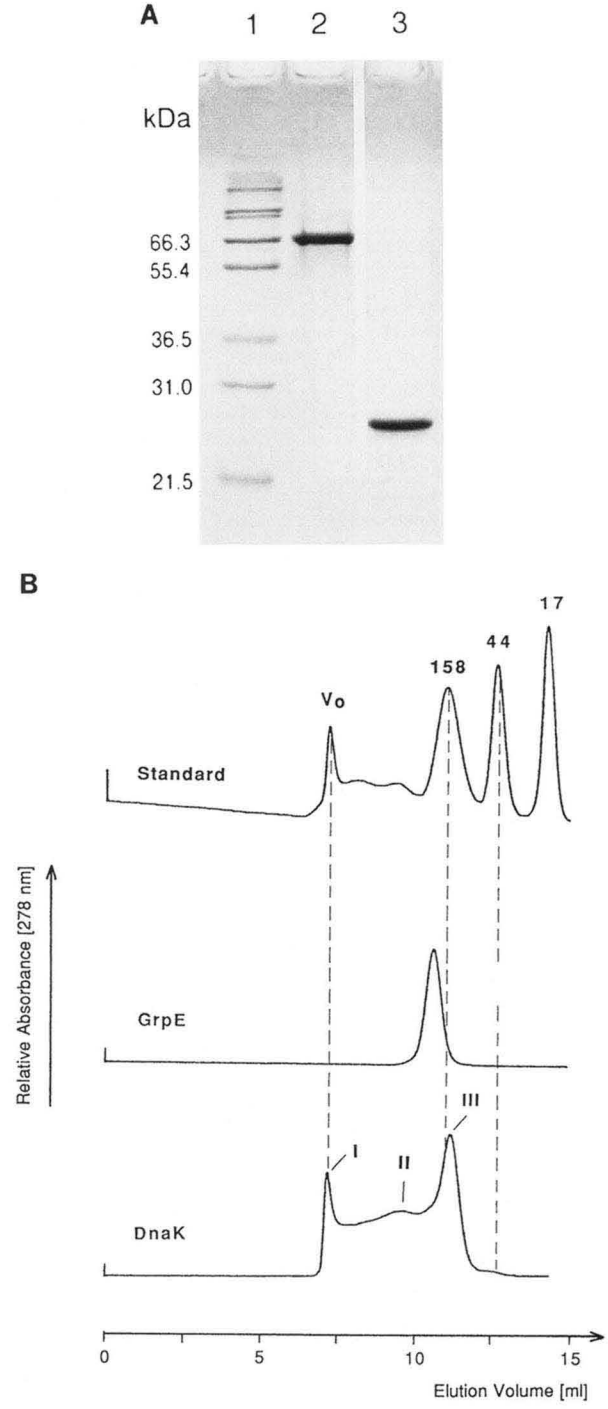


FIG. 1. **SDS-PAGE (*A*) and Superose 12 gel filtration analysis (*B*) of GrpE and DnaK.** *A*: *lane 1*, mixture of standard proteins (soybean trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; porcine lactate dehydrogenase, 36.5 kDa; bovine glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); *lane 2*, DnaK (1.5 μg); *lane 3*, GrpE (1.5 μg). *B*, elution profiles of standard proteins (horse myoglobin, 17 kDa; chicken ovalbumin, 44 kDa; and bovine gamma globulin, 158 kDa; $V_0$ indicates the position of the void volume), GrpE (69 μg), and DnaK (69 μg). Roman numerals *I, II*, and *III* label different peaks of the DnaK chromatogram. *Vertical dashed lines* are drawn in the positions of some peaks for convenient comparisons.

TABLE I
*Hydrodynamic parameters of DnaK, GrpE, and DnaK-GrpE complexes*
Possible models for data fitting are underlined.

| Technique | Parameter | GrpE | DnaK | DnaK-GrpE (1:2) |
|---|---|---|---|---|
| (QLS) | $D_{20,w}$ ($10^{-7}$ cm$^2$ s$^{-1}$) | 4.8–5.6 | 3.4–4.3 | 3.7 |
| Sedimentation velocity | $s_{20,w}$ (S) | 2.7 | 4.0 and 6.3 | 4.8 |
| Mass from Svedberg's equation | Mass (kDa) | 44–51 | 85 and 165 | 117 |
| Sedimentation equilibrium | Mass (kDa) | Single species: 38.0, 40.2[a] | Two species: 67 (75%) and 180 (25%); Monomer-dimer-trimer: 70 (75%) and 140 (12%) and 210 (14%) | Single species: 116, 121[a] |

[a] Values obtained from two independent experiments.

Sedimentation velocity centrifugation of GrpE at the concentration used for QLS experiments yielded a single band with a velocity $s$ of 2.7 S (Table I). Knowledge of the s value as well as the diffusion coefficient determined by QLS permitted usage of the Svedberg equation to calculate a mole mass for GrpE of 44–51 kDa. Sedimentation equilibrium centrifugation of GrpE at the same concentration yielded mole masses for GrpE of 38.0 and 40.2 kDa (Table I).

*Quaternary Structure of DnaK*—When subjected to Superose 12 gel filtration, DnaK eluted with three different maxima: I, II, and III (Fig. 1*B*). Their positions corresponded to apparent mole masses of >2000, 440 and 130 kDa, respectively, relative to globular reference proteins. The relative areas of the different peaks did not vary significantly between different DnaK preparations, but they depended on storage time at 4 °C after thawing, incubation temperature of the sample prior to injection into the column, DnaK concentration, and salt concentration. Peaks I and II increased and peak III decreased with storage time at 4 °C. With respect to the DnaK concentration, at 0.5 mg/ml, peak I was very small, being close to the detection limit, and peak III was the main peak (Fig. 2). By increasing the DnaK concentration up to 16 mg/ml, material in peak III became increasingly shifted to peaks I and II. With respect to the salt concentration, we observed that in the presence of 1 M NaCl there was no material detected corresponding to peak I found at a low salt concentration and the relative positions of peaks II and III were significantly shifted to higher elution volumes, indicating lower hydrodynamic radii of the molecules (elution volumes at 1 M NaCl were corrected for gel shrinking effects by a calibration in the presence of 1 M NaCl).

It is likely that the different forms of DnaK are in equilibrium with each other. To test this possibility, we fractionated the eluate of a gel filtration run with DnaK injected into the Superose 12 column at a concentration of 16 mg/ml. The DnaK concentrations in fractions containing the peak maxima were estimated to be more than 100-fold lower (<0.1 mg/ml) than in the injected sample. After equilibration for about 3 h at room temperature, fractions containing the maxima of peaks I, II, and III were injected into the column. The elution profiles showed mainly a single peak at the position of the original peak III (Fig. 3). Analysis of peak I showed an additional minor peak at the position of former peak II. This additional peak was not observed when the material was equilibrated overnight before analysis (data not shown).

In QLS analysis, DnaK at a concentration of 1 mg/ml showed a broad size distribution yielding diffusion coefficients from 3.4 to 4.3 × $10^{-7}$ cm$^2$ s$^{-1}$. In sedimentation velocity centrifugation, DnaK yielded two bands of 4.0 and 6.3 S (Table I). When these two sedimentation velocities were combined with the upper and lower limits of the diffusion coefficients observed in QLS, mole masses of 85 and 165 kDa were calculated using the Svedberg equation. The DnaK profile observed in equilibrium centrifugation did not fit with a single exponential. When fitted with two exponentials, mole masses of 67 and 180 kDa were obtained with optical density contributions of 75 and 25%, respectively (Table I). Furthermore, the data fitted well with a model assuming coexistence of monomers, dimers and trimers with weight percentages of 75, 12, and 14%, respectively.

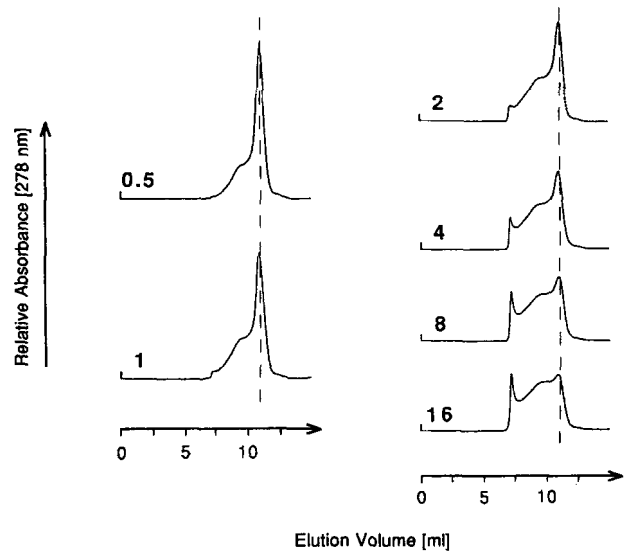


FIG. 2. **Influence of DnaK concentration on gel filtration profile.** A DnaK stock solution (16 mg/ml) was diluted at 20 °C to different end concentrations as indicated by the labels of the profiles in mg/ml. Aliquots of 50 μl of the stock solution and the dilutions were subjected to Superose 12 column chromatography. The reduction of optical densities caused by the dilution factor (relative to the stock solution) was compensated for by a corresponding amplification of the monitor signal. The integral of the total profile is therefore almost constant in every chromatogram. The *vertical dashed line* in the position of DnaK peak III indicates that the positions of corresponding peaks remain constant in all chromatograms.

*Quaternary Structure of DnaK-GrpE Complexes*—To investigate DnaK-GrpE complex formation by gel filtration, DnaK and GrpE were mixed at 1:0.5 to 1:4 molar ratios, equilibrated overnight, and then injected into a Superose 12 column. The corresponding elution profiles (Fig. 4) show that the individual DnaK (*e.g. K(I), K(III)*) and GrpE (*E*) peaks diminished in size when going from DnaK:GrpE ratios of 1:0.5 to 1:1 and a new peak (*C*) appeared. Peak C was positioned between the DnaK peak K(II) and the GrpE peak at an apparent mass of 290 kDa. At the DnaK:GrpE ratio of 1:2, virtually all loaded protein eluted in peak C. When the DnaK:GrpE ratio was further decreased, peak C became progressively asymmetric. At a 1:3 ratio a shoulder was observed at the GrpE position which was even more prominent at a 1:4 ratio. Gel filtration of DnaK and GrpE incubated at 1:2 ratio in the presence of 1 M NaCl also yielded one symmetric peak which, however, was significantly shifted to higher elution volumes compared with the peak C obtained at low salt conditions (not shown).

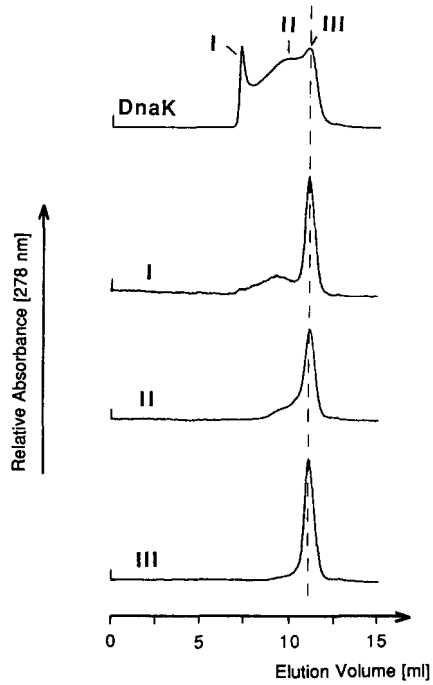


FIG. 3. **Chromatography of different DnaK peaks.** The chromatogram of a DnaK sample at 16 mg/ml (labeled *"DnaK"*) yielding *peaks I, II,* and *III,* and the chromatograms of materials obtained from these peaks after equilibration for 3 h at concentrations of less than 0.1 mg/ml are shown.

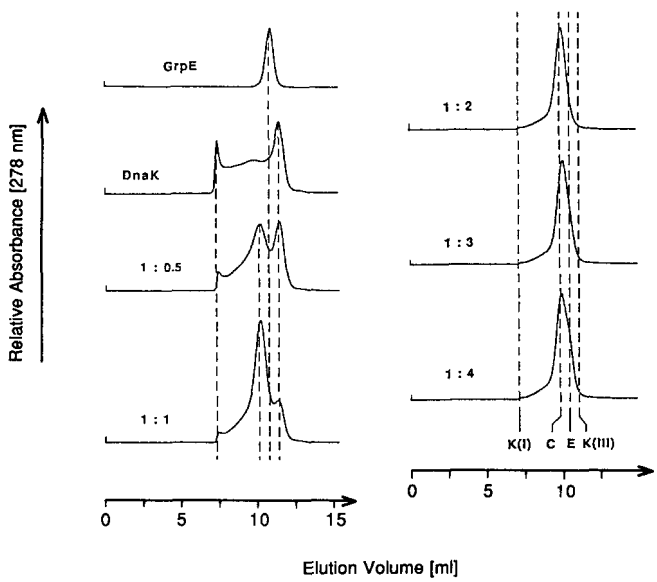


FIG. 4. **Gel filtration analysis of DnaK-GrpE mixtures at different molar ratios.** Samples of 50 $\mu$l at 20 °C containing 2 nmol of GrpE (GrpE), 1 nmol of DnaK (DnaK), or mixtures of 1 nmol of DnaK and 0.5 nmol (1:0.5), 1 nmol (1:1), 2 nmol (1:2), 3 nmol (1:3), and 4 nmol (1:4) of GrpE were injected into a Superose 12 column. The monitor signal was attenuated 2-fold in some cases (1:2, 1:3, and 1:4) leading to a 2-fold reduction of peak sizes. *Vertical dashed lines* are drawn to facilitate comparisons of DnaK peak I (*K(I)*), complex peak (*C*), GrpE peak (*E*) and DnaK peak III (*K(III)*).

For QLS and analytical ultracentrifugation analyses, DnaK and GrpE were mixed at a molar ratio of 1:2 (1.0 mg/ml DnaK and 0.63 mg/ml GrpE) and equilibrated overnight prior to the analyses. In QLS, the size distribution of the mixture was more narrow than that of DnaK alone and yielded a diffusion coefficient of $3.7 \times 10^{-7}$ cm$^2$ s$^{-1}$ (Table I). In sedimentation velocity centrifugation, the proteins exhibited a single migrating band

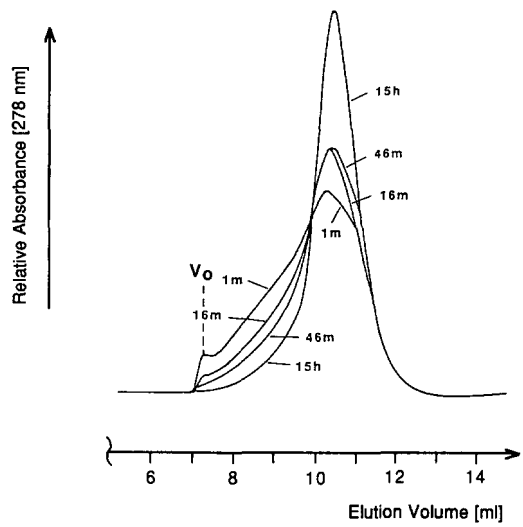


FIG. 5. **Kinetics of DnaK-GrpE complex formation.** Samples of 50 $\mu$l containing DnaK (0.75 nmol) and GrpE (1.5 nmol) were injected at 1 min (*1m*), 16 min (*16m*), 46 min (*46m*) or 15 h (*15h*) after mixing at 20 °C into a Superose 12 column, running at a flow rate of 1 ml/min. The obtained profiles in the peak region are superimposed. $V_0$ indicates the position of the column void volume (identical with the position of DnaK peak I).

with 4.8 S. Using the Svedberg equation we calculated a mole mass of the DnaK-GrpE complex of 117 kDa. In sedimentation equilibrium centrifugation, the equilibrium profile of the complex was fitted to a single exponential, corresponding to a mole mass of 116 kDa (121 kDa, respectively, for an independent second experiment).

*Time Course of GrpE-DnaK Complex Formation*—In order to investigate the time course of DnaK-GrpE complex formation, DnaK and GrpE were mixed at a molar ratio of 1:2 (1.0 mg/ml DnaK and 0.63 mg/ml GrpE), incubated at 20 °C, and injected at different time points into a Superose 12 column. To reduce the time required for gel filtration analysis, the column was operated at a higher flow rate of 1 ml/min, resulting in complete elution of DnaK after 12 min. The elution profiles of the DnaK/GrpE mixture after 1, 16, and 46 min and 15 h of preincubation were determined and are shown in Fig. 5. The individual DnaK and GrpE peaks gradually disappeared with increasing incubation time and peak C of the DnaK-GrpE complex increased at the 290-kDa position (relative to globular reference proteins). More than 1 h of incubation time was required to complete the complex formation. DnaK in the absence of GrpE did not alter its elution profile within the time course of the experiment (data not shown).

Interestingly, although the oligomeric forms of DnaK (peaks I and II) were slowly converted by GrpE to DnaK-GrpE complexes, they had a limited capability of binding GrpE, as revealed by the following experiment. DnaK and GrpE were mixed at a molar ratio of 1:2 but, in order to increase the fraction of oligomeric DnaK, on ice and at higher concentrations (9.2 mg/ml DnaK and 5.8 mg/ml GrpE). After 1 min the mixture was injected into a Superose 12 column operated at high flow rate (1 ml/min), and the eluted fractions were subjected to SDS-PAGE. Coomassie-stained GrpE and DnaK bands were quantified by densitometry, and their ratios were plotted against fraction numbers. GrpE was also detected in fractions containing oligomeric DnaK (Fig. 6*A*). The molar ratio DnaK/GrpE decreased with increasing fraction numbers proportional to elution volumes and approximated the value 1:2 in the region of peak C (Fig. 6*B*).

*Complex Formation of GrpE with the ATPase Domain of DnaK*—GrpE has been reported to interact stable with a 44-

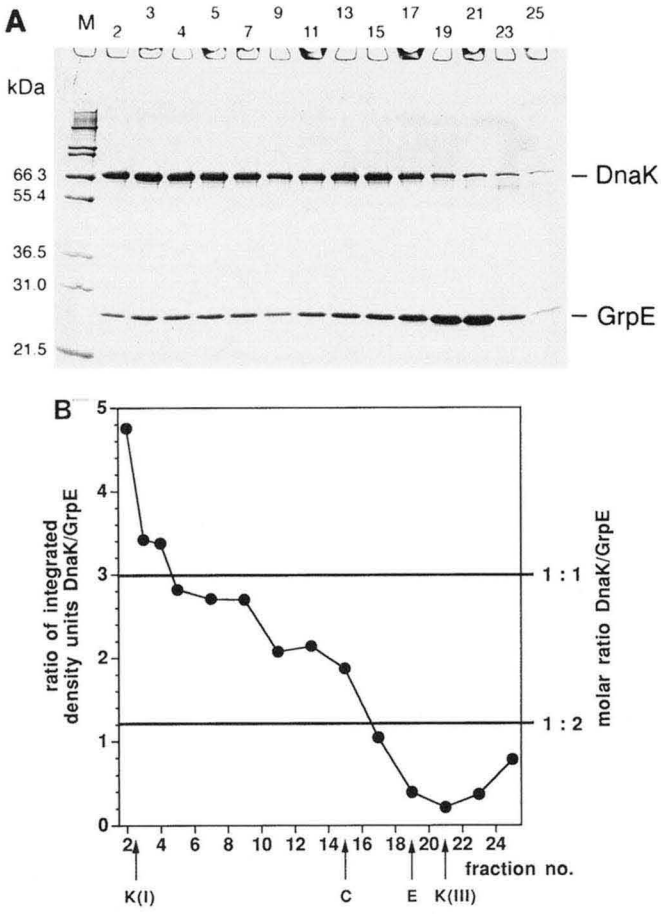


FIG. 6. **The distribution of DnaK and GrpE within the eluate of a fast gel filtration run (1 ml/min) of a DnaK-GrpE mixture at a molar ratio of 1:2 (DnaK:GrpE), injected 1 min after mixing at 0 °C, was analyzed by SDS-PAGE (*A*) and densitometry (*B*).** *A, lane 1* (label "*M*"), mixture of standard proteins (see Fig. 1*A*); *lanes 2–15*, aliquots of fractions of the eluate of the gel filtration run. Lanes are labeled by the fraction numbers. *B*, ratios of integrated DnaK and GrpE bands of different lanes of gel A are plotted against their positions within the elution profiles represented by the fraction numbers. Maxima of DnaK peak I (*K(I)*), complex peak (*C*), GrpE peak (*E*) and DnaK peak III (*K(III)*) were found in fractions 2–3, 15, 19, and 21, respectively, as indicated by the *arrows*. Band integration ratios corresponding to molar DnaK/GrpE ratios of 1:1 and 1:2 were estimated by reference gels and are indicated by *horizontal lines*.

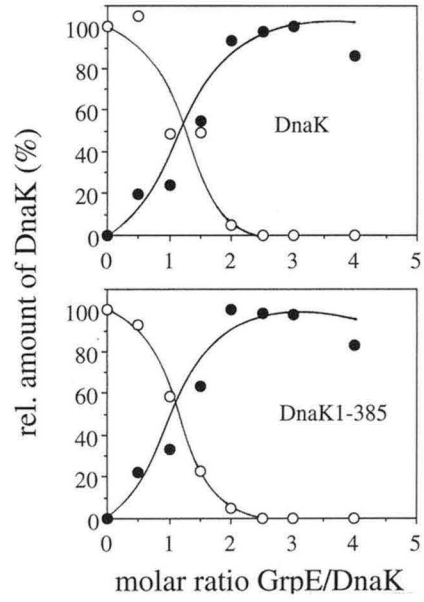


FIG. 7. **Titration DnaK and DnaK1–385 with GrpE.** Full-length DnaK (12 μg; *upper*) and DnaK1–385 (8 μg; *lower*) were incubated for 30 min at 30 °C with amounts of GrpE ranging from 0- to 4-fold molar excess with respect to DnaK/DnaK1–385. The mixtures were analyzed by native PAGE and the relative amounts of free DnaK (●) and GrpE-complexed DnaK (○) in each sample were determined by densitometry and are expressed as percent of total DnaK.

kDa amino-terminal fragment of DnaK (DnaK1–385) comprising the ATPase domain (10). To investigate whether this interaction was similar to the interaction of GrpE with DnaK, we determined the stoichiometry of this complex. DnaK or DnaK1–385 was incubated with different molar ratios of GrpE and complex formation was analyzed by non denaturing PAGE which allowed the differentiation of complexes of GrpE with DnaK or DnaK1–385 from free DnaK and GrpE (10). All GrpE was complexed with DnaK (Fig. 7, *upper*) or DnaK1–385 (Fig. 7, *lower*) when present at an up to 2-fold molar excess over DnaK. Further increases in GrpE concentrations relative to DnaK did not increase the amount of GrpE-DnaK·DnaK1–385 complexes and yielded free GrpE. The behavior of DnaK and DnaK1–385 were indistinguishable with regard to complex formation.

*Absence of DnaK-bound Nucleotides and Presence of a Peptide Substrate Do Not Affect GrpE-DnaK Complex Formation*—Although it is known that GrpE-DnaK complexes are dissociated by ATP (8), it is unclear whether GrpE binding to DnaK requires the presence of nucleotides bound to DnaK. Furthermore, it is unknown whether GrpE-DnaK complex formation is affected by the presence of DnaK substrates. Both questions are relevant to understanding the role of GrpE in the functional cycle of the DnaK chaperone system. Using gel filtration we evaluated the ability of GrpE to form complexes with DnaK that was free of bound nucleotides. Nucleotide-free DnaK and GrpE were incubated for 30 min at 30 °C at a molar ratio of 1:2 and then subjected to Superdex 200 gel filtration. The loaded proteins eluted from the column as a single symmetrical peak corresponding to peak C in Fig. 4, produced by complexes of GrpE with nucleotide-containing DnaK purified by the standard procedure (chromatogram not shown). Incubation of the samples at 30 °C instead of the standard 20 °C used in the experiments shown in Fig. 5 increased the speed and degree of deoligomerization of DnaK.[5]

The effects of the presence of DnaK substrates on GrpE binding were tested by pre incubating DnaK with saturating amounts of the well characterized DnaK/Hsp70 substrate peptide C of vesicular stomatitis virus glycoprotein (17). This 13 -mer peptide binds to DnaK with a $K_d$ of 2.6 μM (13). GrpE was added at a 2-fold molar excess with respect to DnaK. After incubation of the samples for 30 min at 30 °C, they were loaded onto a Superdex 200 column equilibrated in 20 μM peptide C containing running buffer. The obtained chromatogram was not different compared with that obtained from an experiment omitting peptide C during the preincubation of DnaK and in the running buffer, indicating that GrpE-DnaK complexes formed with the same efficiency and characteristics in the presence as in the absence of peptide substrate (chromatograms not shown).

## DISCUSSION

Biochemical and biophysical analyses of the quaternary structures of GrpE and DnaK identified GrpE as a dimer and DnaK as a mixture of monomers, dimers, and higher oligomers which are in a slow equilibrium. DnaK oligomers were dissociated in the presence of GrpE. Furthermore, DnaK-GrpE com-

[5] J. McCarty, B. Bukau, and H.-J. Schönfeld, unpublished data.

plexes were shown to consist of one molecule DnaK and two molecules GrpE and to be formed independently of the presence of peptide substrates or DnaK-bound nucleotides. These results provide novel insight into the structural and functional relationships of this chaperone system.

*GrpE Is Dimeric in Solution*—Determination of the quaternary structure of GrpE was complicated by its nonglobular shape. GrpE migrates in SDS-PAGE significantly slower than expected for a monomer with the mole mass of 22 kDa and elutes in gel filtration at an apparent mole mass of 190 kDa, suggesting octameric conformation in solution. However, mass determination by gel filtration is problematic, since the shape of the analyzed molecule and its interaction with the column support influence its elution behavior (18). We therefore used analytical ultracentrifugation to determine the mole mass of GrpE; this method allows determination of mole masses without using reference proteins for calibration. The sedimentation coefficient determined by ultracentrifugation together with the diffusion coefficient determined using QLS indicated a mass for GrpE of 44–51 kDa, suggesting a dimeric state in solution. This result was confirmed by sedimentation equilibrium centrifugation of GrpE, resulting in mole masses of 38.0 and 40.2 kDa, which are only slightly lower than the 43.6 kDa expected for a GrpE dimer (referred to as $GrpE_2$). The latter methodology does not involve hydrodynamic motion and is considered to provide the most accurate determination of mole mass.

The sedimentation coefficient that we determined ($s_{20,w}$ = 2.7) is in good agreement with the sedimentation coefficient for GrpE obtained from glycerol gradient centrifugation ($s_{20,w}$ = 2.5); however, these data were interpreted by assuming a globular shape for GrpE (calibration with globular reference proteins) and, consequently, a monomeric structure for GrpE was proposed (8). Our results clearly indicate a nonglobular, possibly elongated, shape of $GrpE_2$, since it elutes in gel filtration as a 190-kDa species, its diffusion coefficient is higher and sedimentation velocity is smaller than expected for globular proteins of 44 kDa, and the dissociated molecule migrates slower than expected in SDS-PAGE. Finally, our result agrees with results from glutaraldehyde cross-linking experiments (Ref. 25 as reviewed by Georgopoulos *et al.* (19)).

*The Quaternary Structure of DnaK in Solution Is Heterogeneous*—DnaK appears homogeneous in SDS-PAGE, migrating with the expected monomer mole mass of 69 kDa. The heterogeneity of the quaternary structure of DnaK was indicated by several lines of evidence. In gel filtration, DnaK eluted as multiple peaks. In QLS and analytical ultracentrifugation, the diffusion and sedimentation coefficients were broadly distributed and could not be attributed to a single species. Assuming bimodality, mole masses in the range between monomeric and trimeric DnaK were computed using the Svedberg equation. Furthermore, data obtained from sedimentation equilibrium centrifugation fitted well with models assuming coexistence of monomers and smaller amounts of dimers or dimers and trimers. Ultracentrifugation results led us to conclude that peak III obtained from gel filtration consists of DnaK monomers, peak II mainly of unresolved dimers and trimers, and peak I of higher mole mass forms of DnaK which were not detectable at the concentration of 1 mg/ml used in the ultracentrifugation experiments.

The low peak elution volumes relative to globular reference proteins and the low diffusion coefficients suggested elongated shapes for DnaK molecules. In contrast to the $GrpE_2$ peak, however, DnaK peaks II and III were significantly shifted to higher elution volumes when gel filtration was performed in the presence of high salt concentrations, indicating a more flexible structure for DnaK than for $GrpE_2$.

The different forms of DnaK are in a slow equilibrium for which oligomerization is favored upon increasing the concentration of DnaK. Similar concentration-dependent distributions of oligomeric states of DnaK were found in size exclusion HPLC and nondenaturing gel electrophoresis (20). DnaK oligomers obtained after gel filtration were quantitatively converted to monomers as shown by chromatography. This conversion resulted from establishment of the equilibrium after removal of the monomers and dilution of DnaK by a factor of more than one-hundred during the experiment. The time required for establishment of the equilibrium is long (>1 h) compared with the time needed for fast gel filtration of DnaK (less than 12 min).

*$GrpE_2$ Induces Deoligomerization of DnaK*—The presence of $GrpE_2$ causes dissociation of DnaK oligomers, as revealed by the decrease of DnaK oligomer peak I in the presence of even subequimolar amounts of $GrpE_2$, and by the mole masses derived from equilibrium ultracentrifugation of GrpE/DnaK mixtures which excluded the existence of DnaK oligomers. Dissociation occurs with a slow kinetics similar to that of dissociation of DnaK oligomers upon dilution, suggesting that $GrpE_2$ does not actively disrupt DnaK oligomers but rather stabilizes monomeric DnaK in a heterologous complex. The oligomer dissociating activity of $GrpE_2$ thus may indicate that $GrpE_2$ confers to DnaK a conformation incompatible with stable oligomerization, and it indicates that the heterologous complex was thermodynamically more stable than coexistence of GrpE dimers and DnaK oligomers. However, $GrpE_2$ can also bind to at least a subpopulation of DnaK molecules present in oligomers, since it was found in sub stoichiometric amounts in gel filtration fractions containing DnaK oligomers. Reduced efficiency of $GrpE_2$ binding to DnaK oligomers might have been due to limited accessibility of $GrpE_2$ binding sites within the DnaK oligomers or to lower affinity of $GrpE_2$ for the oligomeric form of DnaK.

*Stoichiometry of the Dnak-GrpE Complex*—The conclusion that the stable complex formed by DnaK and $GrpE_2$ consists of one DnaK and one $GrpE_2$ (DnaK-$GrpE_2$) is based on the following findings. First, in gel filtration chromatography of DnaK-GrpE mixtures the most narrow and symmetrical peak of the complex is obtained in the presence of a 1:2 molar ratio of the components. Second, when DnaK-GrpE mixtures at a molar ratio of 1:2 are analyzed by light scattering or analytical ultracentrifugation a narrow size distribution and a single species was observed. The mole mass of this species was 117 kDa (Svedberg equation) or 116 and 121 kDa (sedimentation equilibrium), which are similar to the expected 113 kDa of a DnaK-$GrpE_2$ complex. The same stoichiometry of 1:2 was observed for complexes between the ATPase domain (DnaK1–385) and GrpE, supporting previous findings (10) and indicating that the regular binding site for $GrpE_2$ is within the ATPase domain of DnaK.

Our experiments identify the GrpE dimer as the functionally active species. Its mode of binding to the ATPase domain of a DnaK monomer remains unclear. A GrpE dimer might have a single DnaK binding site or, alternatively, two binding sites which, however, cannot bind two DnaK molecules simultaneously with high affinity as we did not observe $DnaK_2$-$GrpE_2$ complexes at 1:1 stoichiometry.

*Functional Implications*—The functional cycle of the DnaK chaperone system involves DnaK, GrpE, and DnaJ, the latter not being a subject of the present investigation. Our studies revealed the existence of monomeric, dimeric, and oligomeric DnaK, dimeric GrpE, and DnaK-$GrpE_2$ complexes, raising the number of molecular components involved in the DnaK machinery (21) to at least six. DnaK chaperone activity involves

the binding of unfolded protein (substrate), the folding of this protein and the subsequent release of the folded protein. It is presently unclear what molecular species of the machinery are involved in these different steps of the folding reactions *in vivo* or *in vitro*. We and others showed that in solutions containing only DnaK, the fraction of dimeric and oligomeric DnaK increases with DnaK concentration. There are at least four factors which drive the slow equilibrium of the DnaK oligomers toward monomeric DnaK: the presence of MgATP (20), an increase of temperature,[5] the presence of peptide substrates[6] and the presence of $GrpE_2$. The first three factors were also reported for other members of the Hsp70 family. For example, peptide substrates binding to oligomeric and monomeric forms of BiP yielded exclusively monomeric BiP-substrate complexes at equilibrium (22). Although monomerization of BiP by substrate appears to be comparable with the monomerization of DnaK by $GrpE_2$ that we observed, the molecular mechanisms are probably different. In particular, we showed that $GrpE_2$ binds to the $NH_2$-terminal region of DnaK, whereas peptide substrates bind to C termini of Hsp70 proteins (13, 23, 24).

We suggest that monomeric DnaK is the active molecule in DnaK-mediated protein folding. Oligomeric DnaK would constitute a pool of latent chaperone being activated during heat stress (temperature increase, increasing amounts of unfolded protein, increase of $GrpE_2$ expression (19)).

We showed that $GrpE_2$ also has affinity for a DnaK-substrate complex. This result leads us to suggest that $GrpE_2$ plays a role not only in the named housekeeping of DnaK monomers but also in subsequent interactions involving DnaK; much evidence suggests that this is related to the nucleotide binding by DnaK. GrpE accelerates the release of ATP or ADP bound to DnaK (3). Our data show that $GrpE_2$ was bound to the ATPase domain of DnaK, regardless of whether nucleotide (ADP) was prebound to DnaK or peptide substrate was present. It is therefore possible that $GrpE_2$ binds to DnaK-substrate complexes and dissociates DnaK-bound nucleotides (ADP), functioning as a nucleotide exchange factor. Although $GrpE_2$ remains bound to the DnaK-substrate complexes rebinding of ATP might then induce dissociation of $GrpE_2$ and substrate from DnaK.

Whether the observed stoichiometry of the DnaK-$GrpE_2$ complex has any implications for protein folding *in vitro* and the role of DnaJ are subjects of our present investigations.

[6] J. McCarty and B. Bukau, unpublished data.

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REFERENCES

1. Morimoto, R. I., Tissieres, A., and Georgopoulos, C. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds) pp. 1–30, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J., and Fink, A. L. (1993) *Nature* **365,** 664–666
3. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2874–2878
4. Georgopoulos, C., Liberek, K., Zylicz, M., and Ang, D. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds) pp. 209–249, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) *Nature* **356,** 683–689
6. Schröder, H., Langer, T., Hartl, F. U., and Bukau, B. (1993) *EMBO J.* **12,** 4137–4144
7. Zylicz, M., and Georgopoulos, C. (1984) *J. Biol. Chem.* **259,** 8820–8825
8. Zylicz, M., Ang, D., and Georgopoulos, C. (1987) *J. Biol. Chem.* **262,** 17437–17442
9. Johnson, C., Chandrasekhar, G. N., and Georgopoulos, C. (1989) *J. Bacteriol.* **171,** 1590–1596
10. Buchberger, A., Schröder, H., Büttner, M., Valencia, A., and Bukau, B. (1994) *Nature Struct. Biol.* **1,** 95–101
11. Caspers, P., Stieger, M., and Burn, P. (1994) *Cell. Mol. Biol.* **40,** 635–644
12. Welch, W. J., and Feramisco, J. R. (1985) *Mol. Cell. Biol.* **5,** 1229–1237
13. Buchberger, A., Valencia, A., McMacken, R., Sander, C., and Bukau, B. (1994) *EMBO J.* **13,** 1687–1695
14. Provencher, S. (1982) *Comput. Phys. Commun.* **27,** 229–242
15. Schuck, P. (1994) *Prog. Colloid Polym. Sci.* **94,** 1–13
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685
17. Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989) *Science* **245,** 385–390
18. Schönfeld, H. J., Pöschl, B., Frey, J. R., Loetscher, H., Hunziker, W., Lustig, A., and Zulauf, M. (1991) *J. Biol. Chem.* **266,** 3863–3869
19. Georgopoulos, C., Ang, D., Liberek, K., and Zylicz, M. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds) pp. 191–221, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Palleros, D. R., Reid, K. L., Shi, L., and Fink, A. L. (1993) *FEBS Lett.* **336,** 124–128
21. Georgopoulos, C. (1992) *Trends Biochem. Sci.* **17,** 295–299
22. Blond Elguindi, S., Fourie, A. M., Sambrook, J. F., and Gething, M. J. (1993) *J. Biol. Chem.* **268,** 12730–12735
23. Chappell, T. G., Konforti, B. B., Schmid, S. L., and Rothman, J. E. (1987) *J. Biol. Chem.* **262,** 746–751
24. Palleros, D. R., Shi, L., Reid, K. L., and Fink, A. L. (1994) *J. Biol. Chem.* **269,** 13107–13114
25. Ang, D. (1988) *The Role of the Escherichia coli Heat Shock Protein, grpE, in Escherichia coli growth and λ DNA Replication.* Ph.D. Thesis, University of Utah, Salt Lake City, UT