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Influence of GrpE on DnaK-Substrate Interactions*

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The DnaK chaperone of *Escherichia coli* assists protein folding by an ATP-dependent interaction with short peptide stretches within substrate polypeptides. This interaction is regulated by the DnaJ and GrpE co-chaperones, which stimulate ATP hydrolysis and nucleotide exchange by DnaK, respectively. Furthermore, GrpE has been claimed to trigger substrate release independent of its role as a nucleotide exchange factor. However, we show here that GrpE can accelerate substrate release from DnaK exclusively in the presence of ATP. In addition, GrpE prevented the association of peptide substrates with DnaK through an activity of its N-terminal 33 amino acids. A ternary complex of GrpE, DnaK, and a peptide substrate could be observed only when the peptide binding to DnaK precedes GrpE binding. Furthermore, we demonstrate that GrpE slows down the release of a protein substrate, σ^{32}, from DnaK in the absence of ATP. These findings suggest that the ATP-triggered dissociation of GrpE and substrates from DnaK occurs in a concerted fashion.

Central to the chaperone functions of Hsp70 proteins is the transient interaction of their C-terminal substrate-binding domain with short peptide stretches within substrate polypeptides (1–3). This interaction is controlled by the nucleotide status of the N-terminal ATPase domain of Hsp70, which in turn is regulated by co-chaperones of the DnaJ family and for some Hsp70s by nucleotide exchange factors (4–7). It is still an unsolved question as to why for some Hsp70s nucleotide exchange factors are essential for their activity *in vivo* and *in vitro* while for others nucleotide exchange factors are not essential or do not exist (8–11). It has been shown recently that the nucleotide dissociation rates of Hsp70 proteins vary over a range of almost 3 orders of magnitude (12). The exchange rate of the Hsp70 homologue with the highest exchange rate was nearly as high as the exchange rate of the Hsp70 with the lowest basal exchange rate in the presence of saturating amounts of its exchange factor. This demonstrates that the molecular structure of Hsp70s does not have to be limiting for nucleotide exchange rates and suggests that an optimal exchange rate has been evolved for each system. Therefore, the question arises why nucleotide exchange factors exist and whether they have additional functions.

For the essential nucleotide exchange factor of *Escherichia coli* DnaK (GrpE), it was suggested that it also accelerates substrate release in a nucleotide-independent manner (13). This hypothesis was conceived first on the basis of the unusual structure of GrpE, the N-terminal part of which forms a long α-helix of 100 Å in length (amino acids 34–106) (13). This helix extends well beyond the limits of the ATPase domain, suggesting that it might interact with the substrate-binding domain of DnaK (13). The N-terminal 33 amino acids of GrpE that were unstructured and had to be removed for crystallization were proposed to be responsible for the interaction with the substrate-binding domain of DnaK and for influencing substrate release (13). In a later study (14), it was claimed that even in the absence of added ATP, GrpE was able to stimulate substrate dissociation and association by 200- and 60-fold under saturating conditions. Whereas Mally and Witt (14) found the GrpE-mediated substrate release in the absence of nucleotide and in the presence of ATP, Han and Christen (15) claim that the stimulatory effect only occurs in the presence of ADP and not in the absence of the nucleotide. Finally, it has been suggested that GrpE acts as thermometer, because its activity as a nucleotide exchange factor for DnaK is strongly thermosensitive (16–20). Accordingly, above 45 °C, GrpE would be poorly active, which leads to a stabilization of DnaK-substrate complexes.

To further investigate potential novel functions of GrpE in more detail, we analyzed the effect of GrpE on the binding equilibrium and on the association and dissociation kinetics of the interaction of DnaK with peptide and proteins substrates. In addition, we removed the N-terminal 33 amino acids of GrpE (ΔNGrpE) to analyze the effects of the N terminus on peptide binding and release.

EXPERIMENTAL PROCEDURES

Reagents—Unless indicated otherwise, all of the chemicals were of the highest purity available. ATP-agarose (A2767) and luciferase (L9506) were from Sigma. Luciferin was from Fluka.

Peptides—Peptides σ^{32}-Q132-Q144-C (QRKLFFNLRKTKQC (21)), NR (NRLLLTGC (22)), and vesicular stomatitis virus glycoprotein (VSV-GC)[1] (KLIGVLSSLFRPK (23, 24)) were synthesized chemically using standard chemistry and purified by high pressure liquid chromatography. The peptides were labeled using 2-(4-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (Molecular Probes) (σ^{32}-Q132-Q144-C-AANS), dansyl chloride (D-NR, Molecular Probes), and *N*-succinimidyl-[2,3-^{3}H]propionate (Amersham Biosciences) ([^{3}H]VSV-GC) according to the manufacturer's recommendations and separated from the unre-

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[1] The abbreviations used are: VSV-GC, vesicular stomatitis virus glycoprotein; GdnCl, guanidine chloride; IPTG, isopropyl-1-thio-β-D-galactopyranoside; AANS, 2-(4-anilino)naphthalene-6-sulfonic acid.

acted label by gel filtration on a G25-Sephadex column (5 × 300 mm).

Proteins—DnaK, DnaJ, and GrpE were expressed and purified according to published protocols (25–28). Bound nucleotide was removed as described previously (29). ΔNGrpE was constructed from purified full-length GrpE by digestion with elastase according to Harrison *et al.* (13), purified by anion exchange chromatography and gel filtration as described for full-length GrpE, and verified by electrospray mass spectrometry. σ^{32} was overproduced in a Δ*ftsH* strain (30) and purified as described previously (31). 50–160 μM σ^{32} dialyzed against 50 mM K_2HPO_4/KH_2PO_4, pH 7.6, 200 mM KCl, and 10% glycerol were labeled using *N*-succinimidyl-[2,3-^{3}H]propionate at a 1:1 stoichiometry for 5 h at room temperature. Free label was removed by dialysis against buffer T (20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 200 mM KCl, 2 mM dithiothreitol, 5% glycerol, 0.05% Tween 20). The protein concentration was determined by the Bradford reaction (Bio-Rad protein assay), and the incorporated radioactivity was measured by scintillation counting and the specific activity (0.316 TBq·mmol^{-1}) and labeling efficiency (0.09) was determined.

Substrate Binding and Dissociation Kinetics—Peptide dissociation kinetics using fluorescent-labeled and radiolabeled peptides (σ^{32}-Q132-Q144-C-AANS, D-NR, and [^{3}H]VSV-GC) were performed as described previously (11, 32, 33) with the exception that GrpE was added to the reaction mixture or included with the quench peptide at the indicated concentrations using either a stopped-flow apparatus (SX.18MV, Applied Photophysics) or a PerkinElmer LS-55 spectrofluorometer for the fluorescence measurements. For determination of association kinetics, fluorescent-labeled peptides (0.5 μM) were added to DnaK (0.5 μM) in HKM buffer (25 mM HEPES/KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$) with or without 20 mM K_2HPO_4/KH_2PO_4 in the absence or presence of GrpE at the indicated concentrations. Equilibrium measurements were performed essentially as described (34) by titrating preformed complexes of DnaK (0.5 or 1 μM) with fluorescent-labeled (0.5 μM) or radiolabeled peptides (1 μM) with GrpE. Protein substrate dissociation equilibrium experiments were performed according to a previously published protocol (35). 2 μM [^{3}H]σ^{32} and 1 μM DnaK were incubated in the absence or presence of GrpE (0.25–4 μM $GrpE_2$) in a final volume of 20 μl of buffer T at 30 °C for at least 2 h, and then 90 μl of ice cold buffer T was added and DnaK-bound and free [^{3}H]σ^{32} were separated by gel filtration over a Superdex^{TM} 200 HR 10/30 column (Amersham Bioscience) equilibrated and developed in buffer T. Fractions of 0.5 ml in size were collected, and radioactivity was determined by scintillation counting. For dissociation kinetics, 1 μM [^{3}H]σ^{32} and 1 μM DnaK were incubated in the absence or presence of GrpE (1 μM $GrpE_2$) in a final volume of 20 μl of buffer T at 30 °C for at least 2 h, 5 μM of unlabeled σ^{32} were added, and the reaction mixture was incubated further at 30 °C for the indicated times before analysis by gel filtration as described above.

Refolding of Thermal-denatured Luciferase—Unfolding and refolding were determined as described previously (36) using HKM buffer with 20 mM K_2HPO_4/KH_2PO_4, pH 7.6, and a DnaK:DnaJ:$GrpE_2$:luciferase ratio of 10:2:0.25:1. Luciferase was denatured in the presence or absence of chaperones for 30 min at 42 °C, and refolding was initiated by shifting the reaction mixture to 30 °C.

Circular Dichroism—CD measurements were performed using a Jasco J-715 spectropolarimeter and a cuvette with 0.1-cm path length. Thermal-induced unfolding (15–85 °C) and refolding (85–15 °C) of 1.5 μM $GrpE_2$ or Δ$NGrpE_2$ were followed by continuously monitoring the relative ellipticity at 222 nm (bandwidth, 2 nm) with a scan rate of 30 °C h^{-1}.

GdnCl-induced unfolding of 1.5 μM $GrpE_2$ or Δ$NGrpE_2$ was achieved by a stepwise addition of GdnCl up to 5 M final concentration. Refolding experiments with 1.5 μM $GrpE_2$ or Δ$NGrpE_2$ were performed by dilution series from 5 to 3 M GdnCl and 3 to 0.28 M GdnCl. All of the protein samples were incubated for 30 min at 30 °C before CD measurements took place. For each GdnCl concentration, 10 spectra between 190 and 250 nm (bandwidth, 2 nm) were recorded at a scan rate of 50 nm min^{-1} and the average of the molar ellipticity at 222 nm was plotted against its corresponding GdnCl concentration.

RESULTS

Effects of GrpE on DnaK-Peptide Substrate Complexes at Steady-state Conditions—To investigate the influence of GrpE on the stability of DnaK-substrate complexes, we titrated GrpE to a preformed complex of DnaK·ADP with a peptide, which was labeled at a C-terminal cysteine with 2-(4-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (σ^{32}-Q132-Q144-C-AANS), and measured the fluorescence at steady-state conditions after equilibration of the reaction. As shown in Fig. 1*A*, the fluorescence of the peptide decreases with increasing concentrations of GrpE. This phenomenon could have two explanations. Firstly, GrpE could quench the fluorescent dye, or secondly, GrpE could destabilize the DnaK-substrate complex. To test the first possibility, we repeated the experiment with a radiolabeled peptide ([^{3}H]VSV-GC) and separated DnaK-bound and non-bound peptides afterward by size-exclusion chromatography. This experiment clearly showed that the amount of DnaK-peptide complex decreases with increasing GrpE concentrations (Fig. 1*B*).

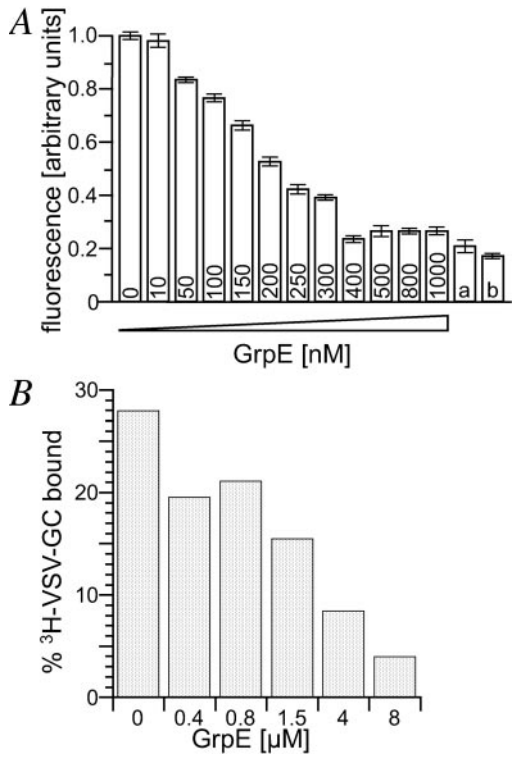

FIG. 1. **GrpE destabilizes DnaK substrate complexes.** *A*, equilibrium titration of GrpE to a preformed complex of DnaK (0.2 μM) and a fluorescent-labeled peptide substrate (σ^{32}-Q132-Q144-C-AANS, 1.0 μM). After incubation for 2 h at 30 °C, fluorescence emission spectra were recorded and the resulting fluorescence maxima (λ_{EXC}, 335 nm; λ_{EM}, 452 nm) were plotted against the corresponding GrpE monomer concentrations. *Bars* denoted with *a* and *b* account for the intrinsic fluorescence of 1.0 μM σ^{32}-Q132-Q144-C-AANS plus 1.0 μM GrpE or 1.0 μM σ^{32}-Q132-Q144-C-AANS, respectively. *B*, equilibrium titration of GrpE to a complex consisting of DnaK (1.0 μM) and a radiolabeled peptide substrate ([^{3}H]VSV-GC, 1.0 μM). The reaction mixtures were incubated for 2 h at 30 °C. Bound and unbound [^{3}H]VSV-GC were separated subsequently by gel filtration. Remaining radioactivities of the DnaK-peptide complexes were plotted against their corresponding GrpE concentrations.

GrpE Has No Effect on the Dissociation of DnaK-Peptide Substrate Complexes in the Absence of ATP—The GrpE-induced decrease in the equilibrium concentration of the DnaK-substrate complex could result either from an increase in the complex dissociation rate as claimed (13–15) or a decrease in the association rate. We therefore determined the dissociation rate constants of the complex of DnaK with σ^{32}-Q132-Q144-C-AANS in the absence and presence of GrpE wild type protein and the ΔNGrpE variant and nucleotides. Surprisingly, neither GrpE nor ΔNGrpE did accelerate the substrate dissociation rate in the absence of nucleotide or in the presence of ADP, which had been purified from contaminating ATP by high pressure liquid chromatography (Fig. 2, *A* and *B*). Only when the DnaK·ADP-substrate complex was mixed with GrpE +

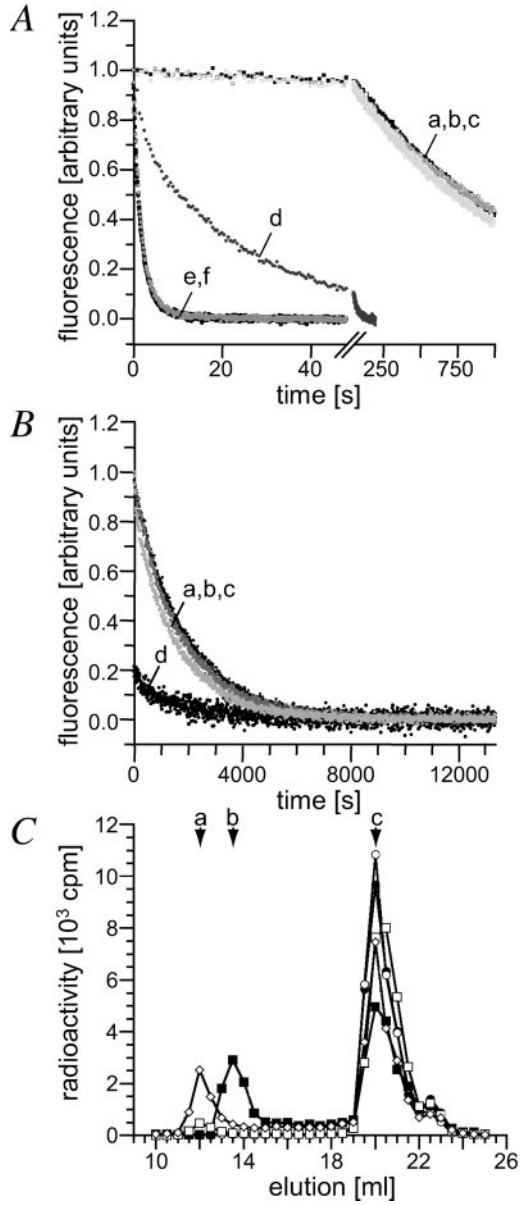

FIG. 2. **GrpE does not accelerate substrate release from DnaK in the absence of ATP.** Dissociation kinetics of a DnaK-peptide complex in the absence or presence of GrpE and ATP were measured using a stopped-flow apparatus or a standard fluorometer. *A*, for the stopped-flow experiments, 0.5 μM DnaK and 0.5 μM of fluorescence-labeled peptide (σ^{32}-Q132-Q144-C-AANS) were incubated for 2 h at 30 °C and mixed rapidly with an unlabeled peptide, σ^{32}-Q132-Q144-C (50 μM) (*a*), or $GrpE_2$ (1 μM) (*b*) or $GrpE_2$ (1 μM) and σ^{32}-Q132-Q144-C (50 μM) (*c*) or ATP (250 μM) and σ^{32}-Q132-Q144-C (50 μM) (*d*) or $GrpE_2$ (1 μM) and ATP (250 μM) (*e*) or $GrpE_2$ (1 μM) and ATP (250 μM) and σ^{32}-Q132-Q144-C (50 μM) (*f*). *B*, for the fluorometer experiments, 0.2 μM DnaK and 0.4 μM of fluorescent-labeled peptide (σ^{32}-Q132-Q144-C-AANS) were incubated for 2 h at 30 °C and mixed subsequently with an unlabeled peptide, σ^{32}-Q132-Q144-C (20 μM) (*a*), or $GrpE_2$ (0.4 μM) (*b*) or $GrpE_2$ (0.4 μM) and σ^{32}-Q132-Q144-C (20 μM) (*c*). For *d*, 0.2 μM DnaK, 0.4 μM σ^{32}-Q132-Q144-C-AANS, and 0.4 μM $GrpE_2$ were incubated for 2 h at 30 °C and then mixed with 20 μM σ^{32}-Q132-Q144-C. *C*, a ternary complex of DnaK with a GrpE dimer and a peptide can be observed by size-exclusion chromatography. DnaK (1 μM) was incubated with [^{3}H]VSV-GC (1 μM) in the absence of GrpE for 2 h at 30 °C. GrpE was added, and the reaction mixture either after 1 min or after 60 min at 30 °C was subjected to gel filtration over a Superdex 200 HR column. *Filled symbols*, no GrpE added; *open symbols*, GrpE added; *filled circle*, [^{3}H]VSV-GC alone; *open circle*, [^{3}H]VSV-GC + GrpE (no DnaK); *filled square*, DnaK + [^{3}H]VSV-GC (no GrpE); *open diamond*, DnaK + [^{3}H]VSV-GC + GrpE after a 1-min incubation; *open square*, DnaK + [^{3}H]VSV-GC + GrpE after a 60-min incubation. *a*, [^{3}H]VSV-GC·$GrpE_2$·DnaK complex. *b*, [^{3}H]VSV-GC·DnaK complex. *c*, free [^{3}H]VSV-GC.

ATP + quench peptide, a significant acceleration of the substrate release was measured (Fig. 2*A*). Identical results were obtained when we used a peptide that was labeled at its N terminus with a dansyl moiety (D-NR) or the radiolabeled peptide [^{3}H]VSV-GC (data not shown).

To directly demonstrate the stability of the ternary complex of a GrpE dimer ($GrpE_2$) with DnaK and a peptide, we performed size-exclusion chromatography on a Superdex 200 HR column, which is able to separate DnaK-peptide and $GrpE_2$·DnaK-peptide complexes (Fig. 2*C*). When GrpE was added to a preformed complex of DnaK with [^{3}H]VSV-GC and then after a 1-min incubation at 30 °C analyzed by gel filtration, comparable amounts of [^{3}H]VSV-GC were found in the ternary complex in the presence of GrpE as in the binary DnaK-peptide complex in the absence of GrpE. When GrpE was present during the incubation of the peptide with DnaK, only minor amounts of radioactivity eluted at the elution volumes of the binary and ternary complexes.

GrpE but Not ΔNGrpE Prevents the Association of Peptide Substrates with DnaK—In contrast, the association of the fluorescent σ^{32}-Q132-Q144-C-AANS peptide with DnaK was influenced significantly by the presence of GrpE but not by the presence of ΔNGrpE. As shown in Fig. 3, the absolute amount of fluorescence that was reached in the plateau phase of the association reaction was decreased with increasing GrpE concentrations. At a 0.5:1 molar ratio of $GrpE_2$ to DnaK, the plateau of fluorescence was ~50% of the value in the absence of GrpE, and at a 1:1 ratio of $GrpE_2$ to DnaK, almost no fluorescence increase was measured. Interestingly, the association rates were not affected by the presence of GrpE (Table I). Similar experiments were repeated with radiolabeled peptides with identical results (not shown). This indicates that GrpE bound to DnaK prevents the association of peptide substrate. It explains why increasing concentration of GrpE led to a decrease in the absolute amount of fluorescence and radioactivity, respectively, observed at equilibrium (see Fig. 1). Together with the gel filtration experiments, these results demonstrate that GrpE significantly slows down peptide association to DnaK and that a ternary $GrpE_2$·DnaK-peptide complex only forms when peptide binds first to DnaK.

Effects of GrpE on the Interaction of DnaK with Protein Substrates—Our data suggest that in the DnaK·$GrpE_2$ complex, the N termini of the GrpE dimer are located in close vicinity to the DnaK substrate-binding cavity. Because the N-terminal 33 amino acids of GrpE are considered to be unstructured, they may in fact bind like a peptide into the substrate-binding cavity of DnaK. If GrpE binds into the substrate-binding pocket, it may inhibit the association of protein substrates to DnaK as well. To test this hypothesis, we incubated radiolabeled *E. coli* heat shock transcription factor σ^{32} with DnaK in the absence and presence of increasing concentrations of $GrpE_2$ and analyzed the formed complexes by size-exclusion chromatography. In the absence of GrpE, two peaks of radioactivity were observed, one at the position of free σ^{32} and one at the position of the σ^{32}·DnaK complex. In the presence of increasing concentrations of GrpE, the peak at the elution volume of the σ^{32}·DnaK complex decreased and an additional peak at lower elution volume appeared (Fig. 4*A*). This peak corresponds to the ternary complex of a GrpE dimer with DnaK and σ^{32}. The peak representing free σ^{32} eluted at exactly the same position in the presence and absence of GrpE. Integration of the radioactivity in all of the peaks showed that the total amount of bound σ^{32} (DnaK·σ^{32} and $GrpE_2$·DnaK·σ^{32} complex) decreased albeit not as dramatically as in the case of the peptides (compare Figs. 1*B* and 4*B*). We concluded from these results that the affinity of DnaK for σ^{32} is reduced as well

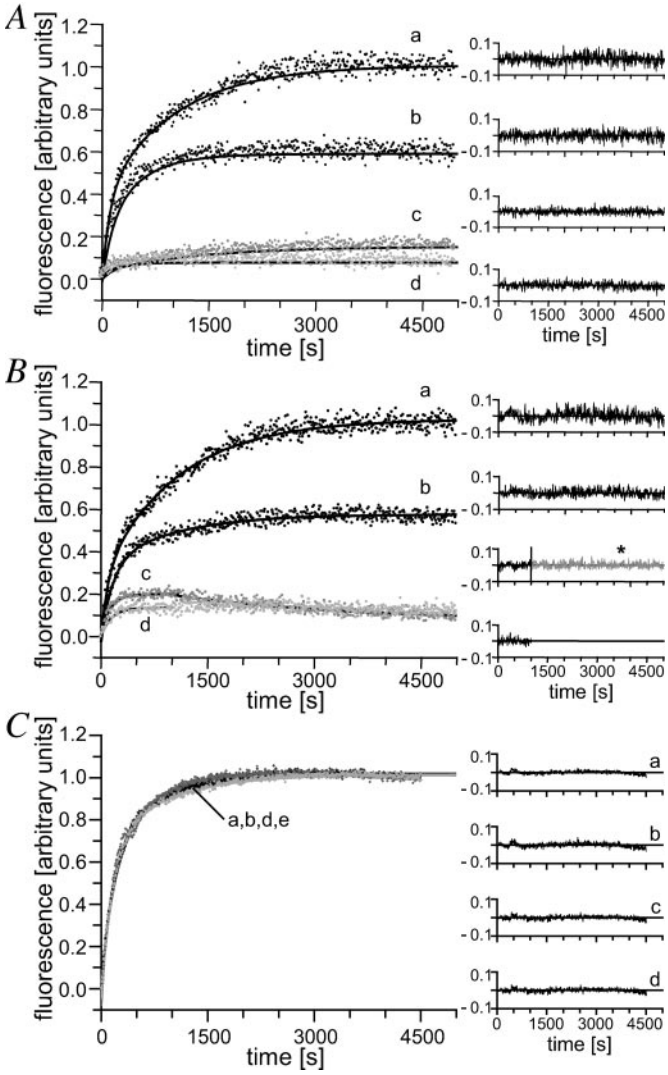

FIG. 3. **The N terminus of GrpE prevents the binding of a peptide substrate to DnaK.** Association kinetics of the fluorescent-labeled peptide substrate σ^{32}-Q132-Q144-C-AANS (0.5 μM) to DnaK (0.4 μM) in the absence or presence of different concentrations (0.0, 0.2, 0.4, and 0.8 μM) of either $GrpE_2$ or the N-terminal deletion variant $\Delta NGrpE_2$. *A*, association of σ^{32}-Q132-Q144-C-AANS to different ratio of unbound DnaK and preformed DnaK·$GrpE_2$ complexes in solution. The final ratio of DnaK and $GrpE_2$ is shown in the *brackets*. *a*, DnaK·$GrpE_2$ (1:0). *b*, DnaK·$GrpE_2$ (1:0.5). *c*, DnaK·$GrpE_2$ (1:1). *d*, DnaK·$GrpE_2$ (1:2). *B,* association of σ^{32}-Q132-Q144-C-AANS (0.5 μM) to DnaK (0.4 μM) by simultaneous mixing of increasing concentrations of $GrpE_2$ (GrpE and σ^{32}-Q132-Q144-C-AANS in one syringe, DnaK in the other). *a*, 0 μM $GrpE_2$. *b*, 0.2 μM $GrpE_2$. *c*, 0.4 μM $GrpE_2$. *d*, 0.8 μM $GrpE_2$. *Asterisk*, residuals shown in *gray* correspond to the decreasing part of the fluorescence trace *c* ($k_{obs} = 9 \times 10^{-4}$ s^{-1}). *C*, association of σ^{32}-Q132-Q144-C-AANS to different ratio of unbound DnaK and preformed DnaK·$\Delta NGrpE_2$ complexes in solution. The final ratio of DnaK and $\Delta NGrpE_2$ is depicted in the *brackets*. *a*, DnaK·$\Delta NGrpE_2$ (1:0). *b*, DnaK·$\Delta NGrpE_2$ (1:0.5). *c*, DnaK·$\Delta NGrpE_2$ (1:1). *d*, DnaK·$\Delta NGrpE_2$ (1:2).

in the presence of GrpE, although not to the same extent as found for peptide substrates.

To investigate this theory in more detail, we determined the dissociation rate of the DnaK·σ^{32} complex in the absence and presence of GrpE. As shown in Fig. 5*A*, the reduction in affinity of DnaK for σ^{32} in the presence of GrpE is not the result of an increase in the dissociation rate. On the contrary, GrpE caused a decrease in the dissociation rate of σ^{32}, yielding a half-life of 58 *versus* 25 min. This increase in half-life was observed independently of the time point when GrpE was added whether it took place at the beginning of the preincubation period together with the labeled [^{3}H]σ^{32} or at time point 0 together with the unlabeled σ^{32}. The only difference observed was that the starting amount of bound σ^{32} was lower when GrpE was added at the beginning of the preincubation, consistent with the previous titration experiment. When ATP was added together with the unlabeled quench σ^{32}, no σ^{32} or very little bound σ^{32} could be detected independent of whether GrpE was present or absent (Fig. 5*B*).

Substrate Binding to DnaK Does Not Affect the Activity of GrpE as Nucleotide Exchange Factor—Together with the results of the ΔNGrpE variant, these data suggest that the N terminus of GrpE is in close vicinity of the substrate-binding pocket. Therefore, we hypothesized that GrpE would be able to monitor the loading status of the substrate-binding domain and that bound substrate could in this way have an influence on the nucleotide exchange activity of GrpE. To test this hypothesis, we determined the GrpE- or ΔNGrpE-accelerated nucleotide exchange rates in the absence and presence of a peptide or protein substrate. For this purpose, we preincubated DnaK with the fluorescently labeled ADP analogue N^8-(4-*N*′-methylanthraniloyl)adenosine-5′-diphosphate-ADP (29) in the absence or presence of the peptide substrate σ^{32}-Q132-Q144-C or the protein substrate σ^{32} for 2 h and then rapidly mixed these complexes in a stopped-flow instrument with unlabeled ATP. No significant differences in the nucleotide exchange rates were observed (data not shown), clearly indicating that the loading status of the substrate-binding domain does not influence the nucleotide exchange function of GrpE.

The close vicinity of the N terminus of GrpE and the substrate that is bound in the substrate-binding domain of DnaK could have an influence on the chaperone function of DnaK. Therefore, we determined the efficiency of DnaK and DnaJ in refolding the thermally denatured firefly luciferase in the presence of GrpE or ΔNGrpE. Luciferase was denatured for 30 min at 42 °C in the presence of DnaK, DnaJ, and either GrpE or ΔNGrpE and subsequently shifted to 30 °C. The loss of activity during denaturation and the regain of activity due to refolding at 30 °C were monitored. No significant differences between GrpE and ΔNGrpE could be detected (Fig. 6).

Expression of grpE-(34–197) Complements the ΔgrpE Temperature Sensitivity Phenotype—To test the functional importance of the N-terminal 33 amino acids *in vivo*, we tried to complement the temperature-sensitive phenotype of a Δ*grpE* strain by ectopically expressing wild type GrpE or ΔNgrpE-encoding genes from an IPTG-inducible promoter (Table II). At 30 °C, the Δ*grpE* strains containing the vector alone or the plasmid encoding ΔNGrpE grew well at all of the IPTG concentrations. In contrast, the strain containing the wild type GrpE-encoding gene did not grow at IPTG concentrations of 100 μM and above. At 42 °C, the Δ*grpE* strain containing the vector alone did not grow. The strain containing the wild type GrpE-encoding gene grew in the absence of IPTG and not at concentrations of IPTG of 100 μM or above. The strain containing the ΔNgrpE-encoding gene only grew in the presence of 100 μM or more IPTG. To better understand these results, we determined the *in vivo* concentrations of GrpE and ΔNGrpE in the Δ*grpE* strain at the different IPTG concentrations through quantitative Western blotting using GrpE-specific antiserum. We found that GrpE was present even in the absence of IPTG, probably because of contaminations of the media with lactose as previously observed in our laboratory on several occasions. At 100 μM IPTG and above, the GrpE concentrations were increased dramatically by >1000-fold over the GrpE level of the wild type strains. This strong overproduction of GrpE explains the lethal phenotype because an excess of GrpE is detrimental to the chaperone function of the DnaK system (34). In contrast, the

TABLE I

The rate of DnaK-peptide association is independent of GrpE

DnaK alone or in complex with GrpE or ΔNGrpE at the indicated ratios was mixed with σ^{32}-Q132-Q144-C-AANS ± GrpE. The increase in fluorescence was fitted to single or double exponential equations. F-Pep, σ^{32}-Q132-Q144-C-AANS; A, amplitude.

	DnaK:$GrpE_2$	$k_{obs,1}$	$k_{obs,1}$	A_1	A_2
		10^{-4} s^{-1}		%	
DnaK + F-Pep		70 ± 12	10 ± 1	30 ± 4	70 ± 4
DnaK·$GrpE_2$ + F-Pep	1:0.5	56 ± 17	11 ± 4	50 ± 12	50 ± 12
DnaK·$GrpE_2$ + F-Pep	1:1	100 ± 24	8 ± 4	42 ± 28	58 ± 28
DnaK·$GrpE_2$ + F-Pep	1:2	48 ± 12	34 ± 16	24 ± 24	76 ± 24
DnaK + F-Pep		115 ± 16	9 ± 2	27 ± 2	73 ± 2
DnaK + $GrpE_2$/F-Pep	1:0.5	64 ± 6	11 ± 1	35 ± 4	65 ± 4
DnaK + $GrpE_2$/F-Pep	1:1	79 ± 8[a]			
DnaK + $GrpE_2$/F-Pep	1:2	70 ± 5[a]			
DnaK + F-Pep		62 ± 1	15 ± 1	58 ± 2	42 ± 2
DnaK·Δ$NGrpE_2$ + F-Pep	1:0.5	71 ± 3	20 ± 1	44 ± 2	56 ± 2
DnaK·Δ$NGrpE_2$ + F-Pep	1:1	105 ± 2	24 ± 1	32 ± 1	68 ± 1
DnaK·Δ$NGrpE_2$ + F-Pep	1:2	60 ± 0.3	12 ± 1	65 ± 1	35 ± 1

[a] single exponential fit.

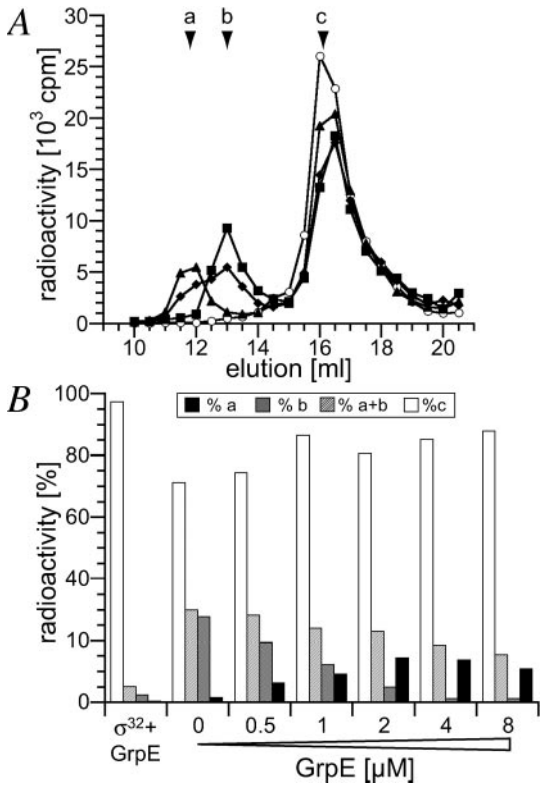

FIG. 4. **GrpE influences the complex of DnaK with heat shock transcription factor σ^{32}.** DnaK, [^{3}H]σ^{32}, and increasing concentration of GrpE were incubated for at least 2 h at 30 °C and subjected subsequently to gel filtration over a Superdex 200 HR 10/30 column. Selected chromatograms, normalized for the total amount of radioactivity injected, are shown in *A*. *Open circle*, [^{3}H]σ^{32} + GrpE; *filled square*, [^{3}H]σ^{32} + DnaK (no GrpE); *filled diamonds*, [^{3}H]σ^{32} + DnaK + 1 μM GrpE; *filled triangles*, [^{3}H]σ^{32} + DnaK + 4 μM GrpE. *B*, the relative amounts of radioactivity under each peak as indicated in *panel A* are plotted against the GrpE concentration.

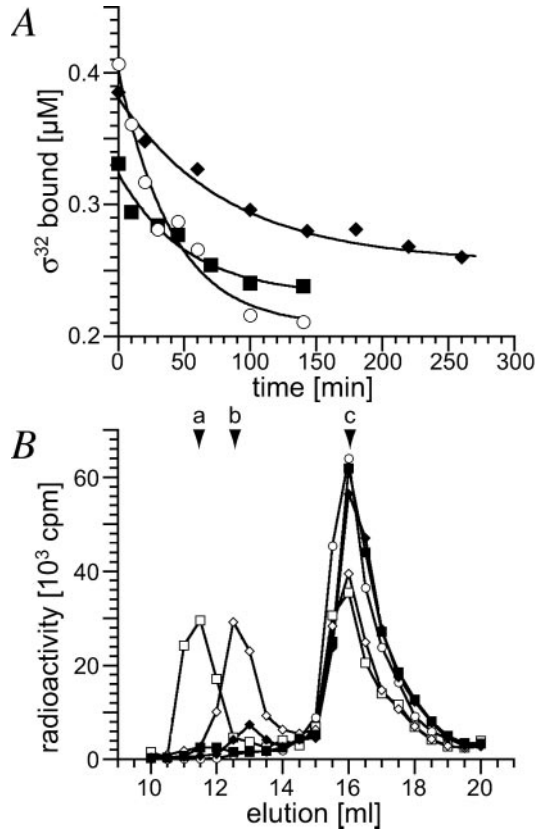

FIG. 5. **GrpE decreases the dissociation rate of the DnaK·σ^{32} complex in the absence of ATP.** *A*, determination of the dissociation rate constants of the DnaK·σ^{32} complex in the absence (*open circles*) and presence of GrpE (*filled symbols*). DnaK (1 μM) and [^{3}H]σ^{32} (1 μM) were incubated in the absence of GrpE (*circles* and *diamonds*) or presence of GrpE (1 μM dimer) for at least 3 h. At time point 0, unlabeled σ^{32} (5 μM) (all *three symbols*) and GrpE (1 μM dimer; *diamonds*) were added and further incubated at 30 °C. DnaK-bound and free σ^{32} were separated by gel filtration. *B*, size-exclusion chromatography experiment as in *panel A* only that ATP (1 mM) was added together with the unlabeled σ^{32}. *Open circle*, [^{3}H]σ^{32} alone; *open diamond*, DnaK + σ^{32} without unlabeled σ^{32}; *filled diamonds*, DnaK + [^{3}H]σ^{32} + unlabeled σ^{32} + ATP after 1 min at 30 °C; *open squares*, DnaK + [^{3}H]σ^{32} + GrpE without unlabeled σ^{32}; *filled squares*, DnaK + [^{3}H]σ^{32} + GrpE + unlabeled σ^{32} + ATP after 1 min at 30 °C. *a*, [^{3}H]σ^{32}·DnaK·GrpE complex. *b*, [^{3}H]σ^{32}·DnaK complex. *c*, free [^{3}H]σ^{32}.

level of ΔNGrpE at 500 μM IPTG was even still lower than the level of wild type GrpE in the absence of IPTG. Therefore, the reason for the difference in their ability to complement the temperature-sensitive phenotype of the Δ*grpE* strain was attributed to the difference of the amount of produced protein and not due to the differences in their biochemical properties.

GrpE Folding Is Positively Influenced by Its N-terminal 34 Residues—Because both *grpE* variants were expressed from the same promoters in identical plasmids and RNA-secondary structure prediction programs did not predict any significant differences in the secondary structure, we hypothesized that the N terminus of GrpE may influence the folding and/or stability of GrpE. To test this hypothesis, we performed thermal and chemical denaturation and renaturation experiments monitoring the folded state of GrpE by circular dichroism and fluorescence spectroscopy. Both methods yielded identical re-

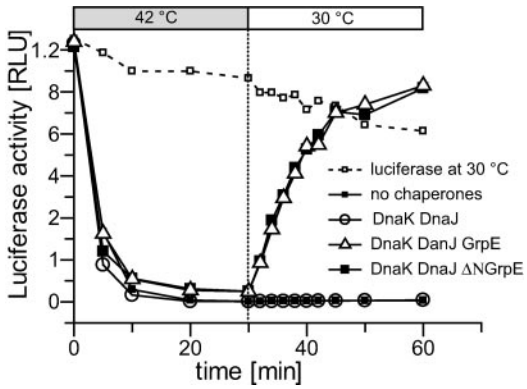

FIG. 6. **The N terminus of GrpE is not required for the potency of the DnaK/DnaJ/GrpE chaperone system during refolding of heat-denatured luciferase.** The activity of firefly luciferase was monitored during the process of heat denaturation (30 min, 42 °C) and refolding by subsequently shifting the temperature from 42 to 30 °C. During unfolding and refolding the final concentrations of luciferase, DnaK, DnaJ, and either $GrpE_2$ or $\Delta NGrpE_2$ were selected as 100, 1000, 200, and 25 nM, respectively.

TABLE II

Complementation of the temperature sensitivity of a Δgrp E strain

MC4100 ΔgrpE was transformed with plasmids expressing *grpE* wild type or Δ*NgrpE* genes. Serial dilutions of overnight cultures were spotted onto LB-agar plates containing the indicated concentration of IPTG and incubated at 30 or 42 °C. +, plating efficiency like vector control at 30 °C, normal colony size; −, plating efficiency at least 4 orders of magnitude lower.

IPTG	30 °C				42 °C			
	0 μM	100 μM	250 μM	500 μM	0 μM	100 μM	250 μM	500 μM
Vector	+	+	+	+	−	−	−	−
grpE	+	−	−	−	+	−	−	−
Δ*NgrpE*	+	+	+	+	−	+	+	+

sults. The denaturation kinetics of GrpE and ΔNGrpE did not exhibit any significant differences. The renaturation kinetics, however, showed clear differences. Whereas full-length GrpE renatured efficiently under the conditions tested, ΔNGrpE renatured much more inefficiently and only a fraction of the original circular dichroism and fluorescence signal could be regained (Fig. 7 and data not shown).

DISCUSSION

Our findings that GrpE and not ΔNGrpE prevents the association of peptides to DnaK while it is capable of binding to a DnaK·peptide complex to form a ternary complex could be explained in two ways. First, the N terminus of GrpE interacts with the substrate-binding domain in a way that the opening of the substrate-binding pocket is prevented. Second, the 33 N-terminal residues are either located in close proximity or directly interact with the substrate-binding pocket. The first hypothesis is unlikely because GrpE had no influence on the dissociation of prebound peptides, which would be expected if GrpE would hamper the substrate-binding domain in its opening movement. The second hypothesis is supported by the fact that the N terminus of GrpE is unstructured and thereby could mimic an unfolded protein. The published algorithm for the identification of DnaK-binding sites does not identify a high affinity-binding site within the N-terminal region of GrpE, in particular, because this region of the protein is rich in negatively charged residues (37). However, a high affinity-binding site might not be necessary because GrpE binds with a K_d of 35 nM to the ATPase domain, and when the N terminus comes in close vicinity of the substrate-binding pocket, even a low affinity-binding site may enter the substrate-binding pocket because of the local high effective concentration of this peptide

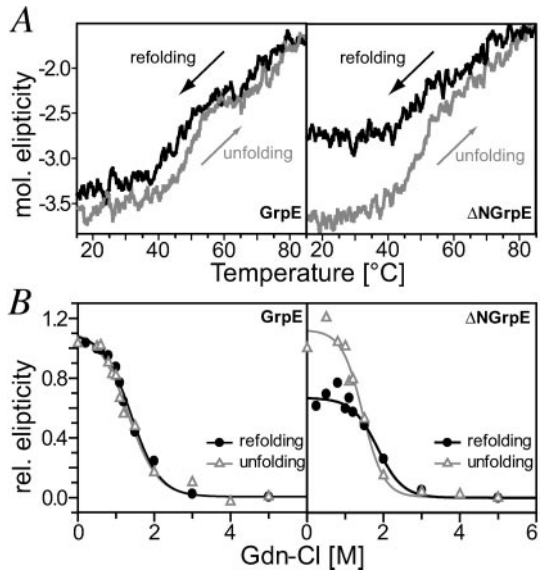

FIG. 7. **ΔNGrpE shows reduce refolding efficiency.** A comparison of the unfolding and refolding behavior of GrpE and ΔNGrpE by monitoring changes in circular dichroism at 222 nm. For thermal unfolding and refolding, samples of 1.5 μM $GrpE_2$ (*A*, *left panel*) or 1.5 μM $\Delta NGrpE_2$ (*A*, *right panel*) were heated from 15 to 85 °C and subsequently cooled from 85 to 15 °C by a scan rate of 1 °C/min. The low concentrations were chosen to avoid aggregation. For GdnCl-induced unfolding and afterward refolding by dilution, circular dichroism spectra (200–270 nM) of 1.5 μM $GrpE_2$ (*B*, *left panel*) and 1.5 μM $\Delta NGrpE_2$ (*B*, right panel) were recorded at the indicated GdnCl concentrations and the resulting values at 222 nm were plotted against the correspondent GdnCl concentration as indicated.

stretch. Consistent with this hypothesis are the results of a recent publication by Witt and co-workers (38) in which they demonstrate that GrpE but not ΔNGrpE interacts with low affinity with a substrate-binding domain fragment of DnaK (DnaK-(393–507); K_d = 67 μM). This interaction with the substrate-binding domain, however, does not contribute significantly to the overall affinity of the GrpE-DnaK interaction because ΔNGrpE binds with similar affinity to DnaK as wild type GrpE and deletion of an exposed loop in the ATPase domain strongly affects the binding of GrpE to DnaK (25, 38). Whether other nucleotide exchange factors of Hsp70s also interact with the substrate-binding domain of their respective partner protein has not been investigated so far.

We and others (35, 39, 40) show that DnaJ also interacts with the substrate-binding pocket of DnaK, thereby competing with substrates, whereas the main ATPase-stimulating interaction occurs in the ATPase domain (41–45). It is intriguing that both co-chaperones that regulate the ATPase cycle of DnaK also interact with the substrate-binding domain. Because ATP binding and hydrolysis induces conformational changes not only in the ATPase domain of DnaK but also in the substrate-binding domain, an interaction of both regulatory cofactors with both domains of DnaK may contribute to the coordination of these conformational changes. *Vice versa*, the conformational change of the substrate-binding domain of DnaK upon association of ATP to its ATPase domain may contribute to the rapid release of GrpE. This might be important given the fact that GrpE binds with high affinity to the ATPase domain and, in the ADP state, has a very low dissociation rate (38). The dissociation rate of GrpE from DnaK upon the addition of ATP has not yet been determined.

A second intriguing finding of our study was that the dissociation rate of the complex of DnaK with a protein substrate was (approximately 2-fold) slowed down significantly in the presence of GrpE. This means that, under the conditions of

high ATP concentrations, the release of the substrate and the release of GrpE are practically always coordinated. Under stress and starvation conditions when the ATP concentration might drop significantly, substrate dissociation is slowed down further through the action of GrpE. Because under these conditions the ATP driven chaperone cycle is not functioning, the release of a misfolded protein substrate would lead to its aggregation with high probability. Similarly, it was shown recently that substrate release from DnaK at a high temperature above 45 °C, when chances for refolding to the native state are also low, is slowed down because of a reversible thermal transition of GrpE, which thereby loses the ability to trigger nucleotide exchange (16–18). Our findings that the N-terminal 33 amino acids are important for the folding of GrpE are consistent with these studies. Dimerization, which is essential for the nucleotide exchange function of GrpE, may start with an interaction of the N-terminal 33 amino acid of both monomers with each other and then proceed through the long α-helices in a zipper-like fashion.

Two recent independent studies claim that GrpE accelerates substrate binding to and the release from DnaK independent of its nucleotide exchange function (14, 15). Our findings are in contrast to these results. We first suspected that our GrpE preparation might have experienced a partial loss of function. Therefore, we carefully tested the functionality of our protein preparations. First, the structural integrity was studied using circular dichroism spectroscopy including the monitoring of thermal and chemical denaturation curves. Second, the nucleotide exchange activity was determined in direct measurements of the ADP and ATP dissociation rates with increasing GrpE concentrations as described earlier (34) or indirectly by determining the steady-state ATPase rates of DnaK in the presence of DnaJ and GrpE. Third, the functionality of GrpE also was shown in chaperone assays by preventing the aggregation and refolding thermally and chemically denatured luciferase. Finally, for our study, we used at least four independent protein preparations. We purified GrpE from ΔdnaK strains to avoid contaminations with DnaK, but we also purified it from a wild type strain in case that DnaK might be important for the folding of GrpE. DnaK was in this case removed by extensive washing with an ATP-containing buffer. To make sure that this GrpE preparation is not contaminated with ATP, we monitored any residual ATP by the very sensitive luciferase assay and found that the contamination is lower than 0.1% of the GrpE concentration. One GrpE preparation was purified in a different laboratory (a kind gift of H.-J. Schönfeld), which also supplied the GrpE that was used in one of the above mentioned studies (15). We could not find any indication that our GrpE preparations are defective and therefore concluded that there must be a different reason for this discrepancy.

The two publications (14, 15) reporting the substrate release function of GrpE are not completely concordant. Whereas Mally and Witt (14) show that GrpE stimulated peptide release in the absence of nucleotide as well as in the presence of ADP, Han and Christen (15) could only find the GrpE stimulation of substrate release in the presence of ADP. Because DnaK has a much higher affinity for ATP (K_D = 1 nM) than for ADP (K_D = 25 nM) (29, 46), small contaminations of the ADP solutions with ATP could explain the results because GrpE would accelerate ADP release and ATP would preferentially bind to DnaK triggering substrate release. We found that commercially available ADP is contaminated usually with 3–5% ATP and therefore always purify our ADP by high performance liquid chromatography. Such a contamination of ADP with ATP, however, could not explain the results of Mally and Witt (14), showing that GrpE stimulated the substrate release from DnaK in the absence of nucleotide. Mally and Witt (14) purified their GrpE by affinity chromatography on immobilized DnaK according to Zylicz *et al.* (47). In this protocol, GrpE is eluted with a buffer containing 10 mM ATP and dialyzed subsequently. If this final dialyzing step is not carried out extensively as described originally, *i.e.* with three buffer changes of sufficient volume, significant amounts of ATP can remain in the GrpE preparation, which would then be able to trigger substrate release by DnaK. Based on these considerations and our own data presented here, we must conclude that GrpE does not have the claimed function to trigger substrate release from DnaK even in the absence of nucleotide. Instead, GrpE decelerates the dissociation of protein substrates in the absence of ATP, and it is likely that a coordinated dissociation of GrpE and substrate occurs when ATP binds to the ATPase domain of DnaK.

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Influence of GrpE on DnaK-Substrate Interactions

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