GrpE N-terminal Domain Contributes to the Interaction with DnaK and Modulates the Dynamics of the Chaperone Substrate Binding Domain

ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · DECEMBER 2007

Impact Factor: 4.33 · DOI: 10.1016/j.jmb.2007.10.002 · Source: PubMed

4 AUTHORS:

Fernando Moro
Universidad del País Vasco / Euskal Herriko…
32 PUBLICATIONS **735** CITATIONS
SEE PROFILE

Stefka G Taneva
Bulgarian Academy of Sciences
96 PUBLICATIONS **858** CITATIONS
SEE PROFILE

Adrian Velazquez-Campoy
University of Zaragoza
119 PUBLICATIONS **2,254** CITATIONS
SEE PROFILE

Arturo Muga
Universidad del País Vasco / Euskal Herriko…
109 PUBLICATIONS **3,159** CITATIONS
SEE PROFILE

doi:10.1016/j.jmb.2007.10.002 J. Mol. Biol. (2007) 374, 1054–1064

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

GrpE N-terminal Domain Contributes to the Interaction with DnaK and Modulates the Dynamics of the Chaperone Substrate Binding Domain

Fernando Moro[1]*, Stefka G. Taneva[1], Adrián Velázquez-Campoy[2] and Arturo Muga[1]*

[1]*Unidad de Biofísica (CSIC/UPV-EHU) y Departamento de Bioquímica y Biología Molecular, Facultad de Ciencia y Tecnología, Universidad del País Vasco, Apartado 644, 48080 Bilbao, Spain*

[2]*Instituto de Biocomputación y Física de Sistemas Complejos, Universidad de Zaragoza, Corona de Aragón 42, 50009 Zaragoza, Spain*

Received 27 July 2007; received in revised form 25 September 2007; accepted 1 October 2007
Available online
5 October 2007

Edited by M. Gottesman

GrpE acts as a nucleotide exchange factor for DnaK, the main Hsp70 protein in bacteria, accelerating ADP/ATP exchange by several orders of magnitude. GrpE is a homodimer, each subunit containing three structural domains: a N-terminal unordered segment, two long coils and a C-terminal globular domain formed by a four-helix bundle, and a β-subdomain. GrpE association to DnaK nucleotide-binding domain involves side-chain and backbone interactions located within the "headpiece" of the cochaperone, which consists of the C-terminal half of the coils, the four-helix bundle and the β-subdomain. However, the role of the GrpE N-terminal region in the interaction with DnaK and the activity of the cochaperone remain controversial. In this study we explore the contribution of this domain to the binding reaction, using the wild-type proteins, two deletion mutants of GrpE ($GrpE_{34–197}$ and $GrpE_{69–197}$) and the isolated DnaK nucleotide-binding domain. Analysis of the thermodynamic binding parameters obtained by isothermal titration calorimetry shows that both GrpE N-terminal segments, 1–33 and 34–68, contribute to the binding reaction. Partial proteolysis and substrate dissociation kinetics also suggest that the N-terminal half of GrpE coils (residues 34–68) interacts with DnaK interdomain linker, regulates the nucleotide exchange activity of the cochaperone and is required to stabilize DnaK–substrate complexes in the ADP-bound conformation.

Keywords: DnaK; GrpE; protein–protein interaction; ITC; binding thermodynamics

Introduction

Hsp70 chaperones are involved in a variety of essential processes for the living cell.[1–3] The function of Hsp70 chaperones is based on their ability to bind short, aggregation-prone, hydrophobic peptide stretches in partially (un)folded polypeptides and release them to allow correct folding. For that, Hsp70 proteins cycle between a high-affinity ADP-bound (R) and a low-affinity ATP-bound (T) conformation. Cycling requires a complex allosteric communication between the nucleotide (NBD) and the substrate-binding (SBD) domains that build up all Hsp70 proteins, which is regulated by the action of cochaperones. Proteins of the Hsp40 family stimulate ATP hydrolysis and consequently the conversion from T to R state, locking the Hsp70 molecule onto the substrate.[4,5] Nucleotide exchange factors (NEFs) facilitate the exchange of bound ADP by ATP that leads to substrate release, bringing the Hsp70 protein back to the T low-affinity state.[6,7] The best characterized Hsp70 system is that of *Escherichia coli*, formed by DnaK (Hsp70), DnaJ (Hsp40) and GrpE (NEF).

GrpE constitutes a functionally conserved family of NEF proteins with representatives in Archaea, Eubacteria and eukaryotic related organelles, such as mitochondria and chloroplasts.[8–10] GrpE is an elongated dimer in solution that consists of a

**Corresponding authors.* E-mail addresses: fernando.moro@ehu.es; arturo.muga@ehu.es.

Abbreviations used: NBD, nucleotide-binding domain; SBD, substrate-binding domain; NR, NRLLLTG peptide; dNR, dansyl-NRLLLTG; ITC, isothermal titration calorimetry; MS, mass spectrometry; wt, wild type; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride.

N-terminal unstructured region of unknown function, a long α-helix that forms two coils in the dimer, a four-helix bundle with two helices from each monomer responsible for protein dimerization and a C-terminal compact β-sheet domain.[11] The nucleotide exchange activity is based on its interaction with $DnaK_{NBD}$ that induces a 14° outward rotation of subdomain IIB, disrupting the nucleotide-binding site and decreasing the affinity for ADP.[11] In the 3-D structure of the $DnaK_{NBD}$:GrpE complex, the dimer is asymmetric and only one monomer interacts with $DnaK_{NBD}$, the contacts being located within the β-sheet and four-helix bundle subdomains. The N-terminal disordered residues and the beginning of the two elongated coils do not participate in the interaction with $DnaK_{NBD}$ and extend well beyond this protein domain. The available crystal structure of the $DnaK_{NBD}$:GrpE complex was obtained with a GrpE mutant harboring a single point substitution, G122D, which greatly reduced its affinity for DnaK, resulting in a deficient phenotype in *Escherichia coli*.[12] Therefore, more detailed studies are required to fully understand the interaction between DnaK and GrpE.

In addition to the nucleotide exchange activity, two different functions have been assigned to GrpE: (i) GrpE and several homologues act as thermosensors of their Hsp70 partners due to a partial or complete reversible unfolding of the molecule under heat shock that reduces its nucleotide exchange rate;[13–15] (ii) GrpE N-terminal residues have been proposed to interact with DnaK, stimulating dissociation of bound substrates in a nucleotide-dependent manner.[11,16–18] The first 33 unordered residues of GrpE might bind as a pseudosubstrate to the DnaK substrate-binding pocket.[19,20] Whether this interaction has a physiological role is at present unknown. In contrast, more recently it has been reported that GrpE stabilizes preformed DnaK–substrate complexes.[19]

Here we report on the energetics of GrpE binding to DnaK as seen by isothermal titration calorimetry (ITC). Papain partial proteolysis and peptide-dissociation kinetics experiments were also carried out to gain a better understanding of the interaction between $DnaK_{SBD}$ and the GrpE N-terminal domain. The role of this GrpE domain was dissected using the deletion mutants $GrpE_{34–197}$ and $GrpE_{69–197}$, as well as $DnaK_{NBD}$. We find that (i) the 1–33 fragment of GrpE interacts as a pseudosubstrate with DnaK as previously proposed[19,20] and (ii) the N-terminal half of the long coils strongly contributes to the binding energetics, interacting most likely with DnaK inter-domain linker and affecting the stability of DnaK–ADP–substrate complexes and nucleotide exchange.

Results

GrpE mutants

The GrpE deletion mutants were designed considering the interactions observed in the 3-D structure of its complex with DnaK (Fig. 1a).[11] Eighteen contacts are found between $DnaK_{NBD}$ and the GrpE proximal monomer, which include nonpolar, polar and ionic interactions. Since the residues involved in these interactions are located within residues 73–197 of GrpE, we constructed the deletion mutant $GrpE_{69–197}$ that should establish the above contacts with $DnaK_{NBD}$ (Fig. 1b). The rest of the elongated N-terminal helices project away from $DnaK_{NBD}$. Residues 1–33 are absent in the crystal structure, but they have been proposed to interact with DnaK in a substrate-like manner.[19] To probe the contribution of this protein domain, $GrpE_{34–197}$ was produced by papain treatment of wild-type (wt) GrpE. The identity of the proteolysis product was confirmed by mass spectrometry (MS). A Coomassie-stained gel containing wt GrpE and the two deletion mutants is shown in Fig. 1c.

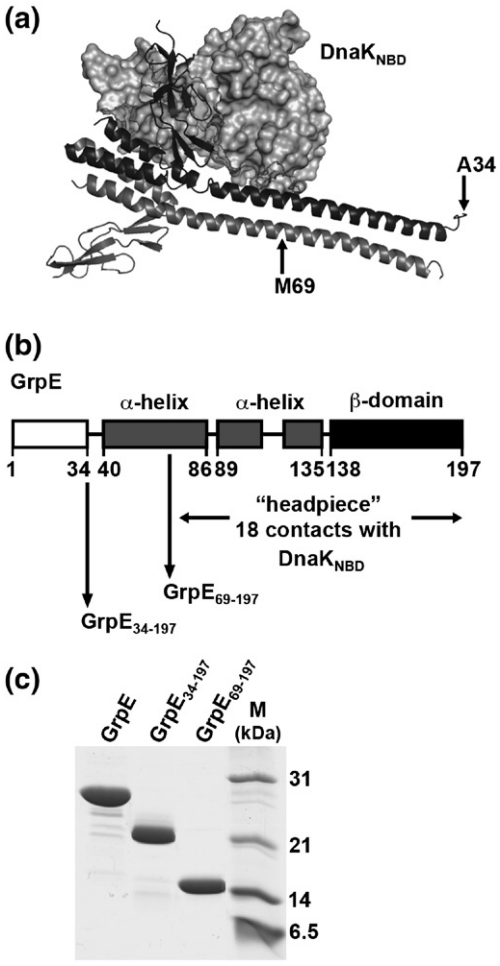

Fig. 1. GrpE deletion mutants used in this study. (a) X-ray structure of GrpE in complex with $DnaK_{NBD}$ (PDB code 1dkg). GrpE is represented as ribbons, and N-terminal residues of each GrpE deletion mutant are indicated. The image was composed with PyMOL. (b) Schematic outline of GrpE monomer domain organization. Fragments deleted in the mutants are indicated. (c) Coomassie-blue-stained gel showing wt GrpE and the mutants $GrpE_{34–198}$ and $GrpE_{69–198}$.

Thermodynamics of GrpE binding to DnaK (ITC)

The calorimetric titration profile for GrpE:DnaK complex formation at 25 °C is shown in Fig. 2a. The enthalpy change is negative (Table 1), indicating an exothermic binding reaction. The binding isotherm and the corresponding fit (Fig. 2b, upper panel) resulted in a stoichiometry close to 1:1 ($N=0.9$) for the GrpE dimer, and a dissociation constant of 157 nM (Table 1). The value of the binding free energy ($\Delta G=-9.4$ kcal/mol) is similar to that estimated by analytical centrifugation experiments.[21] Titration measurements in the absence of ADP yielded a dissociation constant of 52 nM (not shown), indicating, in good agreement with previous results,[11,20] that ADP decreased the affinity of DnaK for GrpE. All subsequent measurements were performed in the presence of 50 μM ADP. Measurements in buffers with different ionization enthalpy (Tris, Pipes and Hepes) resulted in very similar binding enthalpies (not shown), proving that (de) protonation of ionizable residues (acidic, basic or histidine groups) is not involved in the complex formation.[22]

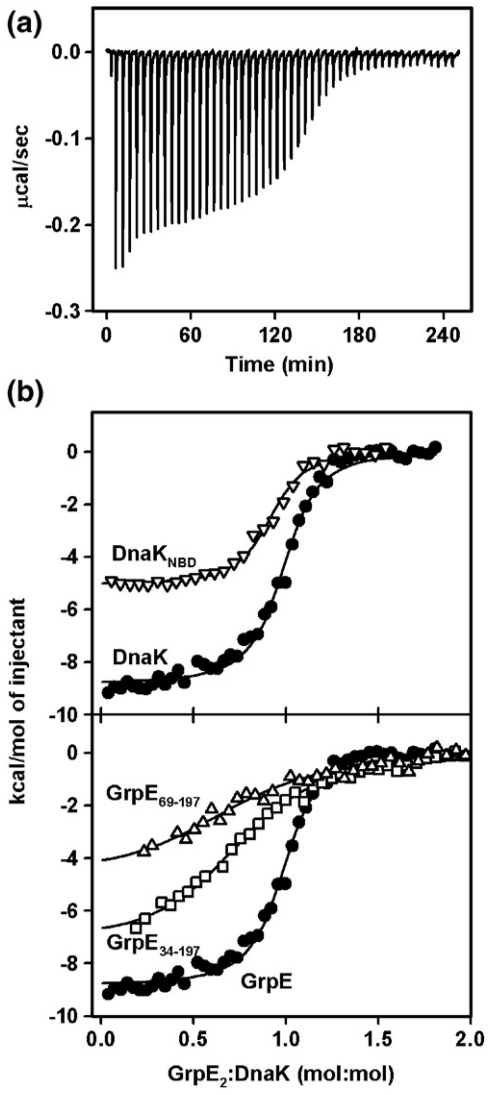

Fig. 2. Isothermal titration of DnaK and $DnaK_{NBD}$ with GrpE and GrpE N-terminal deletion mutants. (a) Baseline-corrected instrumental response of DnaK titration with successive additions of GrpE at 25 °C in 25 mM Hepes (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 2 mM TCEP, 50 μM ADP. (b) Upper panel, integrated isotherms of GrpE binding to DnaK (filled circles) and $DnaK_{NBD}$ (inverted triangles) at 25 °C. Lower panel, binding of GrpE (filled circles), $GrpE_{34–197}$ (squares) and $GrpE_{69–197}$ (triangles) to DnaK at 25 °C. Solid lines represent the best fit to a single-site binding model.

Binding of GrpE to $DnaK_{NBD}$ is also an enthalpically driven process. As compared with wt DnaK, the binding enthalpy decreased from −9.5 to −6.1 kcal mol^{-1} and the dissociation constant slightly increased (Fig. 2b, upper panel, and Table 1). The interaction of wt DnaK with GrpE deletion mutants is shown in Fig. 2b (lower panel). Both GrpE deletion mutants were dimeric, as demonstrated by chemical cross-linking (not shown), in good agreement with published observations,[11,23] and showed binding stoichiometries close to 1 (Table 1). Progressive deletion of the disordered segment (1–33) and the coil N-terminal half (34–68) weakened the interaction with DnaK, as indicated by a six- to ninefold increase in the K_d values. Binding of residues 1–33 to the DnaK peptide-binding site[19] contributes with −1.2 kcal/mol to the affinity for wt GrpE. The binding enthalpy that characterized the interaction between the wt proteins (−9.5 kcal/mol) significantly and progressively decreased as the above-mentioned GrpE regions were deleted, its value dropping to −7.3 and −4.8 kcal/mol for $GrpE_{34–197}$ and $GrpE_{69–197}$, respectively. Since the entropic contribution to DnaK:GrpE and DnaK:$GrpE_{34–197}$ complex formation was close to zero, in these cases the association reaction was mainly driven by enthalpy changes. However, a favorable entropic contribution to the free energy of binding was observed for DnaK:$GrpE_{69–197}$. It is worth mentioning that the overall thermodynamic parameters of DnaK:$GrpE_{69–197}$ and $DnaK_{NBD}$:GrpE complexes were similar.

To get a better understanding of the mechanism of the DnaK:GrpE binding reaction, experiments were performed in the 10–37 °C temperature range, in which conformational changes in the chaperone and cochaperone molecules are not expected to take place.[13,24] The enthalpy change for the binding reaction of the wt proteins is strongly temperature dependent and remains negative at all temperatures over the 10–37 °C range (Fig. 3), resulting in a Δc_p value of −411 cal mol^{-1} K^{-1} (Table 1). This temperature dependence is less pronounced for the binding of $GrpE_{34–197}$ to wt DnaK, with an approximately twofold reduction of the Δc_p value. Interestingly, negligible changes of the binding enthalpy with temperature are observed for DnaK:$GrpE_{69–197}$ and $DnaK_{NBD}$:GrpE complex formation, giving comparably small (close to zero) values of Δc_p (Fig. 3 and Table 1). The thermodynamic parameters for the formation of the different complexes at 25 °C are summarized in Fig. 4 (upper panel).

Table 1. Thermodynamic parameters (ITC)

	N	K_d (μM)	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	ΔG (kcal mol^{-1})	Δc_p (cal mol^{-1} K^{-1})
DnaK:GrpE	0.93±0.09	0.16±0.05	−9.5±0.08	0.1±0.1	−9.4±0.1	−411±7.4
DnaK:GrpE$_{34–197}$	0.8±0.01	1.0±0.1	−7.3±0.2	−0.9±0.2	−8.2±0.05	−245±21
DnaK:GrpE$_{69–197}$	0.8±0.03	1.5±0.3	−4.8±0.7	−2.9±1.0	−7.7±0.1	−29±46
DnaK$_{NBD}$:GrpE	0.95±0.07	0.22±0.09	−6.1±1.2	−3.0±1.2	−9.1±0.25	−38±25
DnaK$_{NBD}$:GrpE$_{69–197}$	0.8±0.2	9.7±0.36	−3.5±0.9	−3.3±0.7	−6.8±0.2	89±40

Titration experiments were carried out by injecting increasing amounts of the desired NEF (GrpE, GrpE$_{34–197}$ or GrpE$_{69–197}$) into an adiabatic cell containing DnaK or DnaK$_{NBD}$ at 25 °C in 25 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 50 mM KCl, 2 mM TCEP, and 50 μM ADP. Binding isotherms were fitted to a single-site binding model with MicroCal Origin software. N is the molar binding stoichiometry referred to GrpE dimer. Δc_p was obtained from the linear plot of ΔH *versus* temperature. Standard deviations are shown.

The lack of a 3-D structure of the complex between the wt proteins hampers a theoretical estimation of the binding enthalpy. The entropic term in the free energy equation $\Delta G = \Delta H - T\Delta S$ of a binding process, where (de)protonation is not involved, can be divided into three contributions:[25]

$$-T\Delta S = -T\Delta S_{sol} - T\Delta S_{conf} - T\Delta S_{tr} \qquad (1)$$

where the solvation term, $-T\Delta S_{sol}$, reflects changes in hydration entropy upon complex formation; the conformational term, $-T\Delta S_{conf}$, accounts for the change in side-chain and backbone conformational entropy associated with the rearrangement of the interacting proteins upon binding; and $-T\Delta S_{tr}$ arises from changes in translational and rotational degrees of freedom of the proteins upon complex formation. The changes in solvation entropy at a given temperature can be estimated by the expression:

$$\Delta S_{sol} = \Delta c_p \ln (T/T^*) \qquad (2)$$

where $T^* = 385.15$ K is the temperature of entropy convergence.[26] The translational and rotational entropy is assumed to be close to the cratic entropy,[27] with a constant value of −7.98 cal mol^{-1} K^{-1} for any binding process with 1:1 stoichiometry. Then ΔS_{conf} can be obtained from Eq. (1) and the experimentally obtained value of ΔS. The values of $-T\Delta S_{sol}$ and $-T\Delta S_{conf}$ for the different complexes are shown in Fig. 4 (lower panel). For the DnaK:GrpE complex, $-T\Delta S_{sol} < 0$, and therefore dehydration of the interacting protein surfaces favors complex formation. The conformational term contributes unfavorably to ΔG, that is, $-T\Delta S_{conf} > 0$, indicating a decrease in conformational degrees of freedom upon interaction of the wt proteins. As the N-terminal segments of GrpE are deleted, the conformational entropy becomes less unfavorable, while the solvation term becomes less favorable. The differences in the conformational entropy contribution can be interpreted as a reduction in the conformational order

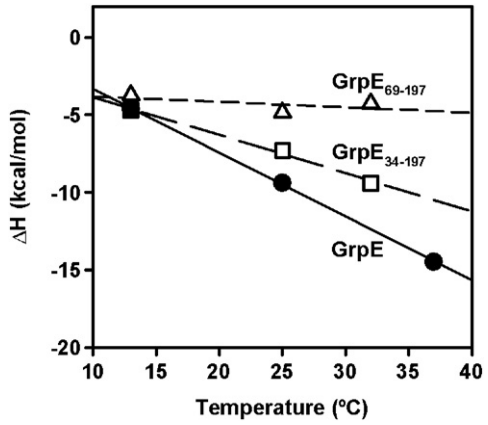

Fig. 3. Temperature dependence of the binding reaction. Binding of GrpE (filled circles), GrpE$_{34–197}$ (squares) and GrpE$_{69–197}$ (triangles) to DnaK was studied at different temperatures as described in Materials and Methods. Δc_p values were obtained from the slopes of the linear regression of the binding enthalpy *versus* temperature for GrpE (continuous line, −411 cal mol^{-1} K^{-1}), GrpE$_{34–197}$ (broken line, −245 cal mol^{-1} K^{-1}) and GrpE$_{69–197}$ (dash lines, −29 cal mol^{-1} K^{-1}).

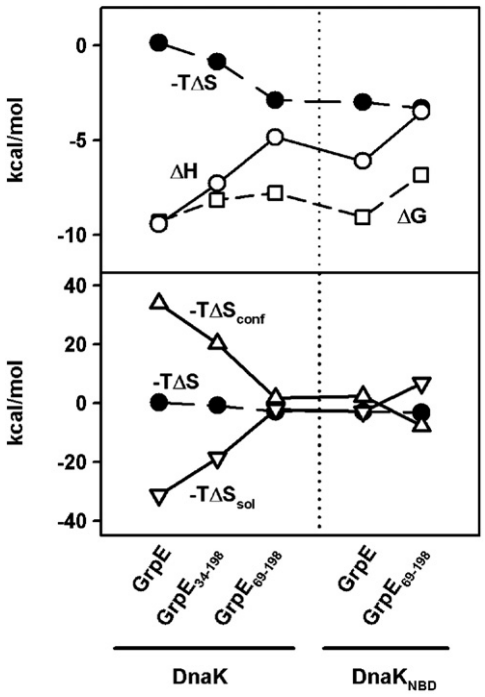

Fig. 4. Thermodynamic parameters. Upper panel, enthalpic (open circles) and entropic (filled circles) contributions to the Gibbs free energy change (squares) for the binding reactions of GrpE, GrpE$_{34–197}$ and GrpE$_{69–197}$ to DnaK or DnaK$_{NBD}$ as indicated. Lower panel, dissection of the entropic contribution (filled circles) into solvation (inverted triangle) and conformational (triangle) terms.

and flexibility of the proteins in the complexes analyzed in the following order:

$$\begin{aligned} &\text{DnaK:GrpE} > \text{DnaK:GrpE}_{34–197} \\ &> \text{DnaK:GrpE}_{69–197} \\ &\approx \text{DnaK}_{\text{NBD}}\text{:GrpE} \\ &\approx \text{DnaK}_{\text{NBD}}\text{:GrpE}_{69–197} \end{aligned}$$

In summary, the unordered first 33 residues and the N-terminal half region of the long paired N-terminal helices of GrpE contribute to complex formation with DnaK, suggesting that both GrpE regions directly interact with the chaperone.

Papain partial proteolysis

An unspecific protease, such as papain, is useful to delimit protein structural domains because its activity on stable secondary and tertiary structures is strongly reduced.[28] Indeed, papain has been successfully used to separate the NBD and SBD of DnaK.[29] DnaK and its complexes with either wt GrpE or the two deletion mutants were digested with papain in the absence or presence of nucleotides (ADP or ATP) (Fig. 5). At low papain concentration (Fig. 5a), nucleotide-free and ADP-bound DnaK were rapidly digested to a lower molecular mass form of approximately 65 kDa (K1) that accounted for around 60% of the total protein (Fig. 5d). MS analysis revealed that this fragment lacks the C-terminal residues from 597 to 638 (Fig. 5c). At a higher papain concentration, fragment K1 represented only 20% of the total protein (Fig. 5b and e). Two other fragments were present when nucleotide-free or ADP-bound DnaK were proteolyzed, their relative abundance increasing with papain concentration: an ~42 kDa fragment (K4) that corresponded, as revealed by MS analysis, to DnaK_{NBD} produced by cleavage at position 386, and a second DnaK_{SBD} fragment that most likely contained residues 387 to 596. In good agreement with this interpretation, Montgomery *et al.*[29] found that the second proteolytic fragment was smaller than recombinant DnaK_{SBD}. Interestingly, cleavage at position 386 was strongly inhibited in the ATP-bound DnaK conformation, with K1 accounting for 80% and 60% of the total protein at low and high papain concentrations, respectively. Additionally, two smaller fragments (K2 and K3) were found and identified by MS as digestions at positions 513 and 526 within helices A and B of DnaK_{SBD}, respectively. The relative amount of both fragments increased with papain concentration, reaching 40% of the total protein at a 1:35 papain:DnaK molar ratio (not shown).

When ADP-bound DnaK was complexed with GrpE or $\text{GrpE}_{34–197}$ before papain treatment, digestion in the vicinity of the linker at residue 386 was strongly inhibited, the abundance of K1 being similar to that found for ATP-bound DnaK (Fig. 5d and e). Residues 1–33 had little effect on the digestion profile of the complex, since under conditions where GrpE was not completely degraded, that is, at the lower papain concentration, the fragment pattern and relative amount of each fragment resembled those found for $\text{GrpE}_{34–197}$ (Fig. 5a and d), the papain final degradation product of wt GrpE (E1). However, as compared with ATP-bound DnaK, cleavage at residues 513 and 526 (fragments K2 and K3, respectively) was diminished, and therefore GrpE or $\text{GrpE}_{34–197}$ binding did not significantly modify the exposure of those sites to papain. The protease sensitivity of $\text{DnaK:GrpE}_{69–197}$ complex was distinct. First, protection of the linker region was reduced at both papain concentrations (Fig. 5a and b), the relative amount of fragments K1 and K4 being intermediate between those estimated for the ADP- and ATP-DnaK conformations (Fig. 5d and e). Second, the accessibility of cleavage sites at helices A and B of DnaK_{SBD} in the presence of $\text{GrpE}_{69–197}$ was increased to a level similar to that found for ATP-bound DnaK. At the higher papain concentration, $\text{GrpE}_{69–197}$ was partially degraded to a lower molecular mass band that, as identified by MS, corresponded to $\text{GrpE}_{78–197}$ (E2), the degradation product accounting for half of the initially added cochaperone. $\text{GrpE}_{69–197}$ partial degradation did not modify the exposure of DnaK linker, since papain treatment of ADP-bound DnaK complexed with $\text{GrpE}_{78–197}$ generated a similar amount of fragment K4 to that found for $\text{GrpE}_{69–197}$ at both papain concentrations (Fig. 5f).

Taken together, these results indicate that the N-terminal half of the long GrpE helices, residues 34–68, protects the DnaK linker from protease attack, and that this GrpE region is required to maintain the DnaK_{SBD} in an ADP-like conformation, since its deletion increases exposure of papain digestion sites at helices A and B. As already mentioned, residues 1–33 have little effect on the above conformational changes.

Substrate dissociation kinetics

To determine the effect of deletions at the GrpE N terminus on the stability of DnaK–substrate complexes, peptide dissociation kinetics was followed under different experimental conditions. First, we examined the release of dansyl-labeled NRLLLTG peptide (dNR) from nucleotide-free DnaK (Fig. 6a, continuous lines). Addition of nonfluorescent NR produced a slow dissociation of dNR with a k_{off} value of 0.72×10^{-3} s^{-1}, as previously described.[16] GrpE alone dissociated DnaK-bound peptide with similar kinetics that was not modified by the simultaneous addition of NR (Table 2), suggesting that GrpE competes with bound peptide as a pseudosubstrate. Deletion of residues 1–33 strongly reduced the k_{off} (Table 2), indicating that this GrpE region was indeed competing with bound dNR for the binding site. $\text{GrpE}_{69–197}$ showed faster dissociation kinetics than $\text{GrpE}_{34–197}$, although both lack residues 1–33. Second, in the presence of ADP, GrpE but not quench NR induced a small increase in k_{off} (Fig. 6a and Table 2). Combination of quench NR and GrpE resulted in a synergic effect that further increased the k_{off} value.

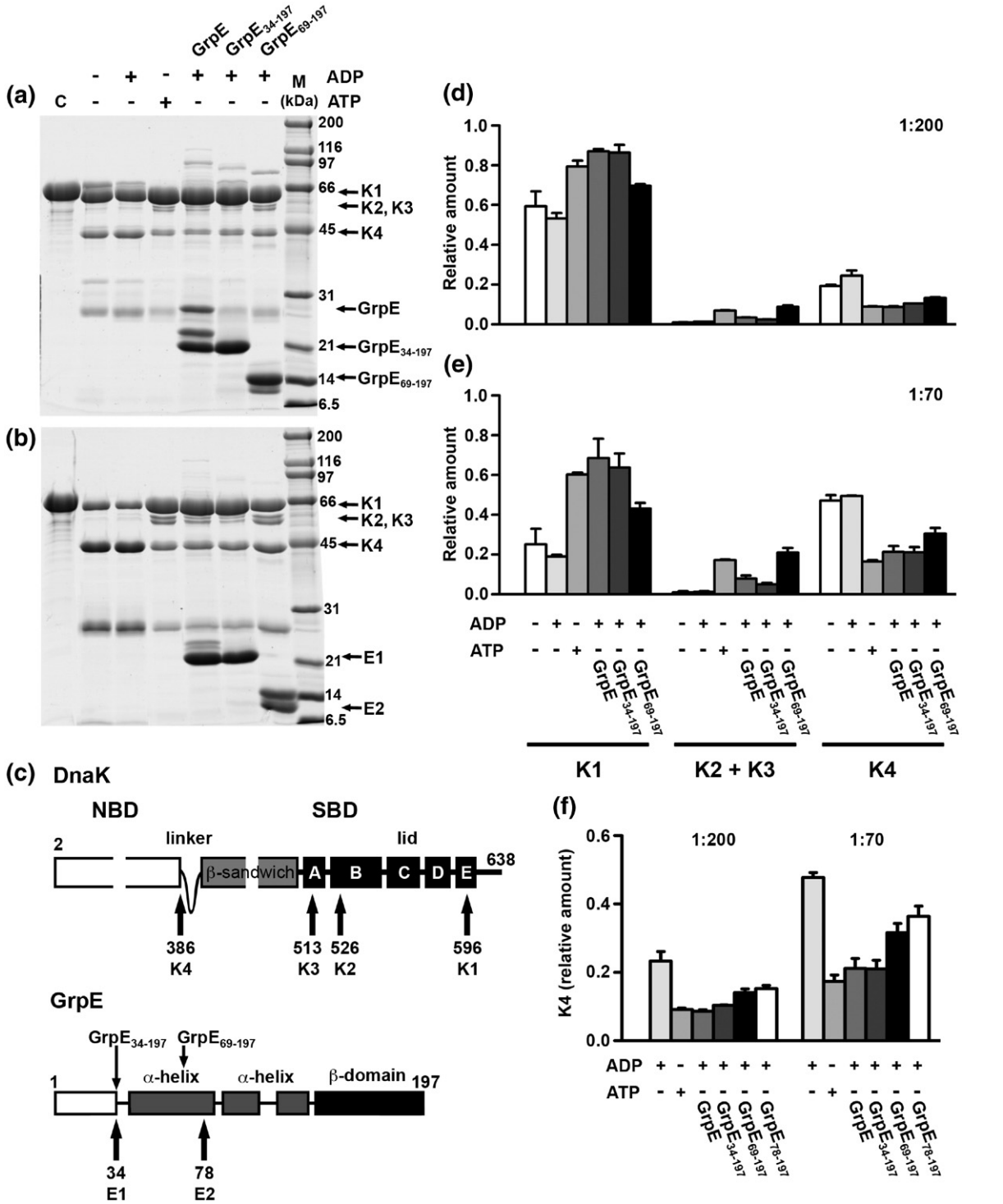

Fig. 5. Papain partial proteolysis of DnaK. (a, b) Coomassie-stained SDS-PAGE of papain digestion products of DnaK (20 μM) in the absence or presence of nucleotides (2.5 mM), and 30 μM GrpE, $GrpE_{34–197}$ or $GrpE_{69–197}$, as indicated in each lane. Papain:DnaK molar ratio was 1:200 (a) or 1:70 (b), and digestion was performed for 90 min at 25 °C. Reactions were stopped by addition of 100 μM E-64 inhibitor. Lane C, control of undigested DnaK. (c) Schematic outline of DnaK and GrpE domains. Papain digestion sites that give rise to K1, K2, K3 and K4 fragments, as identified by MS, are indicated at their corresponding positions. (d, e) Amount of fragments K1, K2, K3 and K4 relative to total DnaK at 1:200 (d) and 1:70 (e) papain:DnaK molar ratios. Coomassie-stained gels were scanned and bands were quantified by densitometry in a G-800 Calibrated Densitometer (BioRad). Values are the average of three independent experiments. (f) Amount of K4 fragment generated at 1:200 and 1:70 papain:DnaK molar ratios in the presence of $GrpE_{78–197}$. Papain digestion and quantification are performed as above.

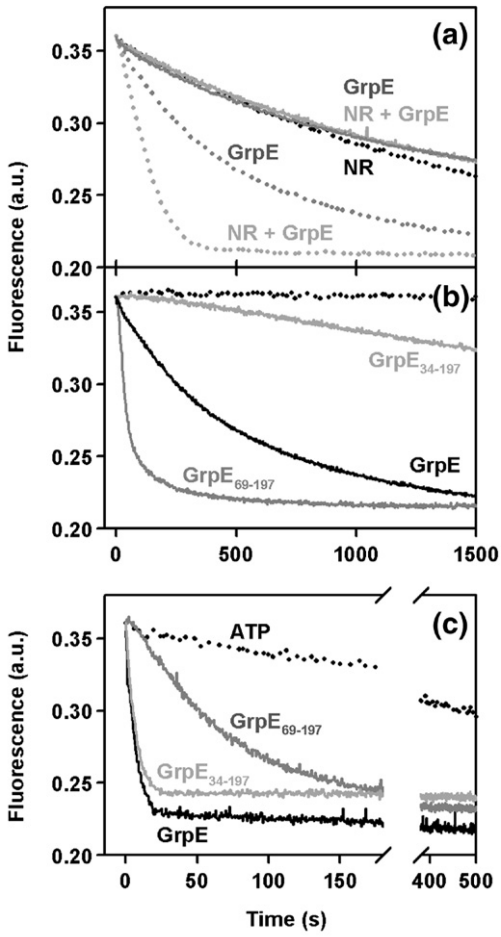

Fig. 6. Substrate release kinetics. (a) DnaK–substrate complexes were preformed by incubation of DnaK (1 μM) with the fluorescent peptide dNR (1 μM) for 50 min at 25 °C in the absence (continuous lines) or the presence of 0.5 mM ADP (dotted lines). Peptide dissociation was induced by addition of nonfluorescent 50 μM NR (black lines), 2 μM GrpE (dark gray lines) or both (light gray lines) to a vigorously agitated cuvette. Dissociation was followed by monitoring the decrease of dansyl moiety fluorescence at 535 nm. Curves were fitted to single or double exponentials (see Table 2). (b) Dissociation of DnaK–dNR complexes in the presence of ADP by GrpE (black continuous line), $GrpE_{34–197}$ (light gray continuous line) or $GrpE_{69–197}$ (dark gray continuous line) at a 1:2 molar ratio versus DnaK. A control experiment after addition of buffer is also shown (dotted line). (c) Preformed DnaK–ADP–dNR complexes as in A were dissociated by vigorous mixing with 50 μM ATP alone (black dotted line) or with GrpE (black continuous line), $GrpE_{34–197}$ (light gray continuous line) or $GrpE_{69–197}$ (dark gray continuous line). GrpE and GrpE mutant concentration was 0.1 μM.

Under these conditions, $GrpE_{34–197}$ still showed a slow dissociation of bound dNR (Fig. 6b) that, similarly to wt GrpE, was accelerated by the simultaneous addition of quench NR. $GrpE_{69–197}$ strongly stimulated peptide dissociation from ADP–DnaK (Fig. 6b), the kinetics following a double-exponential decay with a major (approximately 90% of the amplitude) fast component of 29.8×10^{-3} s^{-1}, one order of magnitude faster than the k_{off} value obtained for GrpE-induced dissociation of the DnaK–ADP–dNR complex. Interestingly, addition of quench NR did not significantly increase substrate release by $GrpE_{69–197}$ (Table 2). These results indicate that the interaction of the GrpE "headpiece," residues 69–197, with DnaK promotes substrate dissociation, while the N-terminal half of the long GrpE helices, segment 34–68, compensates for this effect, thus stabilizing the DnaK–peptide complex. Residues 1–33 may act as a pseudosubstrate for the peptide-binding site, competing with bound peptides.

Finally, the ATP-induced dissociation kinetics of ADP–DnaK–peptide complexes was characterized. As expected, GrpE greatly accelerated nucleotide exchange and, consequently, peptide release (Table 2). $GrpE_{34–197}$ exhibited a similar behavior with comparable k_{off} values. However, the kinetics was around twofold slower for $GrpE_{69–197}$. To better demonstrate this effect and bring the k_{off} values within an experimentally reliable range, we reduced the GrpE:DnaK molar ratio to 0.1:1 (Fig. 6c), a situation that might be closer to that found *in vivo*.[30] Lowering the GrpE:DnaK molar ratio induced a slight reduction (2.7-fold) in the k_{off} values for GrpE and $GrpE_{34–197}$, whereas that for $GrpE_{69–197}$ decreased one order of magnitude (Table 2). Under these conditions, nucleotide exchange should be rate limiting and govern the rate of peptide release. These data suggest that deletion of the N-terminal half of the long helices reduced the nucleotide exchange activity of $GrpE_{69–197}$, as previously shown for similar GrpE deletion mutants.[18,31]

Table 2. DnaK–substrate complex release kinetic constants

		$k_{off}\times10^3$ (s^{-1})		
	Ratio[a]	–	+NR	+ATP
DnaK–dNR				
–		–	0.72±0.01	n.d.
+GrpE	1:2	0.80±0.01	0.81±0.01	n.d.
$+GrpE_{34–197}$	1:2	0.13±0.02	0.53±0.01	n.d.
$+GrpE_{69–197}$	1:2	0.78±0.01	1.03±0.02	n.d.
DnaK–ADP–dNR				
–		–	0.62±0.01	1.9±0.1
+GrpE	1:2	2.7±0.3 (0.6±0.2)[b]	7.6±0.1	410±67
$+GrpE_{34–197}$	1:2	0.17±0.01	3.8±0.01	405±38
$+GrpE_{69–197}$	1:2	29.8±3.2 (3.8±0.3)[b]	34.4±4.2	185±59
+GrpE	1:0.1	n.d.	n.d.	148±10
$+GrpE_{34–197}$	1:0.1	n.d.	n.d.	152±6
$+GrpE_{69–197}$	1:0.1	n.d.	n.d.	14.4±2.3

Preformed DnaK–dNR complexes in the absence or presence of ADP (0.5 mM) were rapidly mixed with GrpE or $GrpE_{34–197}$ or $GrpE_{69–197}$ alone or combined with quench NR (50 μM) or ATP (50 μM). Dissociation kinetics were followed and fitted to single or double exponentials when indicated. k_{off} values are the average of at least three independent experiments. Standard deviations are given.

[a] DnaK:GrpE/$GrpE_{34–197}$/$GrpE_{69–197}$ final ratio in the complex.

[b] Curves were best fitted to double exponentials with a minor second order component (in parenthesis). In both cases, the minor component accounted for less than 10% of the amplitude. n.d. not determined.

Discussion

The role of GrpE in the dissociation of DnaK–substrate complexes has been interpreted assuming the interaction of its N-terminal domain with $DnaK_{SBD}$.[11,16–20] Here we have dissected this interaction, using two GrpE deletion mutants, $GrpE_{34–197}$ and $GrpE_{69–197}$, the latter containing all the residues involved in the stabilization of the $DnaK_{NBD}$:GrpE complex.[11] No structural information is available on the possible interaction that the N-terminal GrpE region (residues 1–68) might establish with $DnaK_{SBD}$, since this chaperone domain is absent in the 3-D structure of the complex (Fig. 1a). Our ITC data show that similar thermodynamic parameters are obtained for $DnaK_{NBD}$:GrpE, DnaK:$GrpE_{69–197}$ and $DnaK_{NBD}$:$GrpE_{69–197}$ complex formation, the main difference being the higher affinity of the former. Therefore, in the absence of $DnaK_{SBD}$ or GrpE N-terminal residues 1–69, complex formation would involve similar interactions. However, binding of wt GrpE to DnaK displays distinct properties: (i) a higher exothermicity, that is, a larger negative binding enthalpy, (ii) an almost negligible entropic contribution to the binding reaction at 25 °C; and (iii) a larger Δc_p for the association reaction, as compared to the above complexes. It is unlikely that the observed differences between the wt and mutant complexes are due to indirect effects of the deletions on the interacting protein domains, since similar binding parameters are obtained for complexes that either contain DnaK ($DnaK_{NBD}$:GrpE) or GrpE (DnaK:$GrpE_{69–197}$), or both proteins mutated ($DnaK_{NBD}$:$GrpE_{69–197}$). Decomposition of the entropic contribution to the binding reaction ($-T\Delta S$) shows that as GrpE segments 1–33 and 34–68 are deleted the solvation term becomes less favorable, suggesting that the area involved in complex formation progressively decreases. However, for the same protein complexes, the conformational term is lowered; that is, taking wt GrpE as a reference, we obtain $-T\Delta\Delta S_{conf}$ values of −13.7 and −32.2 kcal/mol for $GrpE_{34–197}$ and $GrpE_{69–197}$, respectively. Assuming that each residue experiences a conformational entropy loss such as in a protein folding process of about 0.5 kcal/mol,[32] our results would suggest that interaction of the GrpE N-terminus with $DnaK_{SBD}$ could induce ordering of around 60 residues. Therefore, these data suggest that a direct interaction between residues 1–68 of GrpE and DnaK occurs, which significantly contributes to the binding process and involves a conformational rearrangement of the interacting proteins.

The GrpE N terminus can be divided into two regions, namely, an N-terminal stretch, comprising residues 1–33, that adopts an unordered conformation, and the segment 34–68 that constitutes the N-terminal half of the long helical coils of the protein (Fig. 1b). Our data might help to get further insight into the DnaK regions that interact with GrpE fragments 1–33 and 34–68, and the nature of the conformational changes associated to complex formation. We found that GrpE promotes a slow dissociation of a fluorescent peptide bound to DnaK, with similar kinetics to that obtained upon addition of excess unlabeled peptide (Fig. 6a and Table 1), as already reported.[17,19] Substrate dissociation is slowed after deletion of the N-terminal unordered residues in $GrpE_{34–197}$, a finding compatible with previous reports showing that this GrpE region interacts with the substrate-binding pocket of DnaK as a pseudosubstrate.[19,20] Although this GrpE domain is negatively charged and lacks a typical hydrophobic stretch with high affinity for DnaK, the close vicinity and high local concentration might allow this interaction. It should be mentioned that the effect of the N-terminal residues of GrpE on peptide–DnaK–ADP complex dissociation is not significant when compared with the dissociation signal brought about by ATP, which is at least two orders of magnitude faster. Indeed, we observed DnaK–substrate complex dissociation kinetics in the presence of GrpE similar to those found by Brehmer *et al.*[19] and substantially smaller than those shown in previous reports.[16,17] As has been discussed, ATP contaminations in commercial ADP (as high as 4%, as we observed by ion exchange chromatography) or in purified GrpE samples might accelerate substrate dissociation,[19] and thus, ADP[33] and GrpE[18] should be purified to avoid such a contamination, as done in this work.

Partial proteolysis with papain shows that the presence of the N-terminal half of the GrpE coils (residues 34–68) promotes a strong protection against protease cleavage of a site adjacent to the DnaK interdomain linker, as does ATP binding, suggesting that in the complex this region of the long GrpE helices is in close proximity to the linker. DnaK interdomain linker participates in the allosteric communication and becomes solvent protected upon ATP binding, as shown by an amide hydrogen exchange/MS approach and a recent NMR study,[34,35] and thus less accessible to papain. The observed protection of DnaK linker by the GrpE 34–68 fragment might be due to a direct interaction with this DnaK region or to an indirect ATP-like conformational change brought about by complex formation. Both interpretations are not exclusive, and with the present data we cannot rule out any of them. Our data suggest that the interaction of the GrpE segment 34–68 with DnaK pursues a twofold function: first, it is required for the stabilization of DnaK–substrate complexes, evidenced by the increased substrate dissociation kinetics promoted by the headpiece mutant $GrpE_{69–197}$. This mutant induces an ATP-like exposure of proteolytic sites at the DnaK lid, suggesting an opening of the DnaK lid that would enhance substrate dissociation. Binding of GrpE induces changes in DnaK intrinsic fluorescence, which suggest a chaperone conformation intermediate between the ADP- and ATP-bound DnaK states.[17] Considering also that DnaK fluorescence spectral properties depend on conformational changes of helices A and B in $DnaK_{SBD}$,[36] these observations suggest that GrpE promotes a conformational change in the lid subdomain of ADP-

bound DnaK. Moreover, the interaction of the long coils with DnaK maintains the binding pocket closed, stabilizing DnaK–substrate complexes. Second, the GrpE 34–68 fragment ensures optimal nucleotide exchange activity, as suggested by the lower ATP-induced peptide dissociation rate observed for $GrpE_{69–197}$ when compared to wt GrpE and $GrpE_{34–197}$ (Fig. 6c). Slower nucleotide exchange rates were also found for GrpE deletion mutants lacking the complete coil region.[18,31] Such GrpE deletion mutants are dimeric and well folded with a high α-helical content as observed by CD[18] and a single denaturation transition centered around 70 °C followed by differential scanning calorimetry.[23] We found a similar differential scanning calorimetry denaturation profile for $GrpE_{69–197}$ (T_m = 72 °C; not shown), suggesting that the deficient activity of N-terminal deletion mutants is not due to folding problems. Remarkably, Gelinas *et al.*[31] found that a chimeric GrpE in which the long coils were replaced by the coiled-coil domain of the yeast transcription factor GCN4, a substitution that has been shown to be functional for different proteins,[37,38] was also deficient in nucleotide exchange. This finding reflects the specificity of the GrpE N-terminal sequence in the interaction with DnaK. Interestingly, the thermosensor function of GrpE is based on the reversible thermal unfolding of the long coils that promotes slower nucleotide exchange rates at heat shock temperatures.[13,39] The defective activity of coil deletion mutants might mimic the effect of thermal denaturation of this protein region. These data indicate that the interaction of the 34–68 GrpE segment with DnaK regulates the nucleotide exchange activity of the cochaperone.

Our data suggest that GrpE interacts with both domains of DnaK and that the complex formation induces conformational rearrangements of both proteins. Note that GrpE does not bind to a construct containing the linker and the complete $DnaK_{SBD}$,[40] and therefore its interaction with $DnaK_{NBD}$ is essential for the former to occur. The interaction of GrpE with both DnaK domains might also induce an approximation of these domains as suggested by fluorescence spectroscopy, a situation that resembles the ATP-induced domain docking recently described.[35] N-terminal residues of two chloroplast GrpE isoforms have been recently implicated in the interaction with their Hsp70 partner.[41] Interacting with both DnaK domains, the cochaperone GrpE would concomitantly stabilize DnaK–substrate complexes and control the ATP/ADP exchange rate, achieving a subtle tuning of the allosteric signal.

Materials and Methods

Protein purification

DnaK and $DnaK_{NBD}$ were purified as described.[36] GrpE was purified following the protocol described by Mehl *et al.*[18] to avoid the use of ATP during purification. $GrpE_{34–197}$ was obtained by digestion of wt GrpE with papain, 125:1 (w:w) for 90 min at 25 °C, and further purified in a HiLoad Superdex 75 16/60 column (GE Healthcare) equilibrated in 20 mM Hepes (pH 7.6), 50 mM KCl and 5% glycerol. The sequence of the resulting protein was confirmed by MS and N-terminal sequencing. $GrpE_{69–197}$ was cloned in pET3a vector (Novagen) between NdeI/BamHI sites using the primers GGAATTCCATATGGAAAACCTGCGTCGT and CCCAGATCTTTAAGCTTTTGCTTTCGCTAC. $GrpE_{69–197}$ was purified by a modified protocol. In brief, transformed BL21(DE3) cells were grown to exponential phase, induced with 1 mM IPTG and harvested. Lysis was achieved by sonication in buffer A (25 mM Hepes, pH 7.6, 0.5 mM EDTA, 1 mM DTT, 5% glycerol) plus 1 mM PMSF, and cell debris was removed by ultracentrifugation. Lysates were subjected to ammonium sulfate fractionation at 30% and 55%. The final pellet was dissolved in 10 ml of buffer A, and after overnight dialysis was loaded onto a 50-ml Affi-gel Blue (Biorad) column equilibrated in buffer A. The bound protein was eluted with a 400-ml linear gradient to 1 M NaCl. Fractions containing $GrpE_{69–197}$ were concentrated and loaded onto a HiLoad Q-Sepharose 16/10 HP column (GE Healthcare) equilibrated in buffer A that was eluted with a 100-ml linear gradient to 1 M NaCl. Finally, $GrpE_{69–198}$ was loaded onto a HiLoad Superdex 75 16/60 column (GE Healthcare) equilibrated in and eluted with 120 ml of buffer A. The purified protein was concentrated and frozen at −70 °C. $GrpE_{78–198}$ was obtained by papain treatment of $GrpE_{69–198}$, 70:1 (w:w). After a 90-min digestion, the reaction was stopped by addition of 50 μM E-64 and excess inhibitor was removed in a PD10 column.

GrpE, $GrpE_{34–197}$ and $GrpE_{69–197}$ concentrations were determined by the Biorad protein assay and expressed in moles of dimer.

Isothermal titration calorimetry

ITC measurements were carried out with a VP-ITC MicroCalorimeter (MicroCal, Inc., Northampton, MA). Titration experiments were performed in 25 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 50 mM KCl, 2 mM TCEP, 50 μM ADP. Proteins were extensively dialyzed against the above buffer. DnaK or $DnaK_{NBD}$ (12–15 μM) in the calorimetric cell was titrated by successive injections of GrpE, $GrpE_{34–197}$ or $GrpE_{69–197}$ (120–135 μM). Similar injections in buffer were performed to determine the heat of dilution used to correct the experimental data. The binding isotherms, ΔH *versus* molar ratio, were analyzed with a single-site binding model using MicroCal Origin software. The calorimetric enthalpy of binding, ΔH, and association constant, k_a ($K_d = 1/k_a$), were directly obtained from the fit. The free energy (ΔG) and the entropy (ΔS) of binding were derived from the basic thermodynamic expressions: $\Delta G = -RT \ln k_a = \Delta H - T\Delta S$, where R and T are the gas constant and the absolute temperature, respectively. To determine the changes in the heat capacity, Δc_p, for the different complexes, binding experiments were carried out at several temperatures in the range 10–37 °C. Δc_p was estimated from the slope of a linear plot of ΔH *versus* T.

Papain partial proteolysis

Partial papain digestion was performed at 25 °C in 20 mM Hepes (pH 7.6), 50 mM KCl and 2.5 mM $MgCl_2$. Papain stocks were dissolved in 20 mM Hepes (pH 7.6), 50 mM KCl and activated by addition of 50 mM β-mercaptoethanol and incubation at 37 °C for 30 min.

DnaK (20 μM) was incubated with 2.5 mM ADP, ATP, or without nucleotide for 5 min at 25 °C. GrpE, $GrpE_{34–197}$ or $GrpE_{69–197}$ (30 μM) was added and incubated for 10 min to form stable complexes. Proteolysis was initiated by addition of papain from the activated stocks at 1:200 and 1:70 papain:DnaK molar ratios. The reaction was left for 90 min at 25 °C and stopped by addition of 100 μM E-64 (Sigma). Proteolysis products were analyzed by SDS-PAGE (12.5% gels) followed by staining with Coomassie Brilliant Blue and densitometry in a G-800 Calibrated Densitometer (BioRad).

Peptide release kinetics

DnaK (1 μM) was incubated with the fluorescent peptide dNR (1 μM) for 50 min at 25 °C in 20 mM Hepes (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$ and 0.5 mM ADP to form stable DnaK–substrate complexes. ADP (Sigma) was purified as described.[33] Peptide dissociation was achieved by addition of 50 μM ATP or nonfluorescent NRLLLTG peptide (NR), in combination with wt GrpE or GrpE deletion mutants when desired. DnaK to wt or mutant GrpE molar ratios were 1:2 or 1:0.1. Fluorescence of the dansyl probe in dNR was continuously measured on a SLM8100 spectrofluorimeter (Aminco) with excitation at 335 nm, emission at 535 nm, and excitation and emission slit widths of 4 and 8 nm, respectively. NR and dNR peptides were purchased from Neosystem (Strasbourg, France).

Acknowledgements

This work was supported by the Ministerio de Educación y Ciencia (Grant BFU2004-03452/BMC), the Universidad del País Vasco (Grants UPV 13505/2001 and GIU06/48), and Diputación Foral de Bizkaia (Grant DIPE06/20). S.G.T. is a visiting professor at the University of Basque Country and associate member of the Institute of Biophysics, Bulgarian Academy of Sciences. A.V.-C. is supported by the Ministerio de Educación y Ciencia (Grant SAF2004-07722) and a Ramón y Cajal contract. F.M. is supported by a Ramón y Cajal contract. We thank Kerman Aloria for the MS analysis.

References

1. Hartl, F. U. & Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, **295**, 1852–1885.
2. Mayer, M. P. & Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol. Life Sci.* **62**, 670–684.
3. Bukau, B., Weissman, J. & Horwich, A. (2006). Molecular chaperones and protein quality control. *Cell*, **125**, 443–451.
4. Schröder, H., Langer, T., Hartl, F. U. & Bukau, B. (1993). DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* **12**, 4137–4144.
5. Cyr, D. M., Langer, T. & Douglas, M. G. (1994). DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem. Sci.* **19**, 176–181.
6. Packschies, L., Theyssen, H., Buchberger, A., Bukau, B., Goody, R. S. & Reinstein, J. (1997). GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. *Biochemistry*, **36**, 3417–3422.
7. Hohfeld, J. & Jentsch, S. (1997). GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. *EMBO J.* **16**, 6209–6216.
8. Groemping, Y., Klostermeier, D., Herrmann, C., Veit, T., Seidel, R. & Reinstein, J. (2001). Regulation of ATPase and chaperone cycle of DnaK from *Thermus thermophilus* by the nucleotide exchange factor GrpE. *J. Mol. Biol.* **305**, 1173–1183.
9. Miao, B., Davis, J. E. & Craig, E. A. (1997). Mge1 functions as a nucleotide release factor for Ssc1, a mitochondrial Hsp70 of *Saccharomyces cerevisiae*. *J. Mol. Biol.* **265**, 541–552.
10. Schroda, M., Vallon, O., Whitelegge, J. P., Beck, C. F. & Wollman, F. A. (2001). The chloroplastic GrpE homolog of *Chlamydomonas*: two isoforms generated by differential splicing. *Plant Cell*, **13**, 2823–2829.
11. Harrison, C. J., Hayer-Hartl, M., Di Liberto, M., Hartl, F. U. & Kuriyan, J. (1997). Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science*, **276**, 431–435.
12. Grimshaw, J. P., Siegenthaler, R. K., Züger, S., Schönfeld, H. J., Z'graggen, B. R. & Christen, P. (2005). The heat-sensitive *Escherichia coli* grpE280 phenotype: impaired interaction of GrpE(G122D) with DnaK. *J. Mol. Biol.* **353**, 888–896.
13. Grimshaw, J. P., Jelesarov, I., Schönfeld, H. J. & Christen, P. (2001). Reversible thermal transition in GrpE, the nucleotide exchange factor of the DnaK heat-shock system. *J. Biol. Chem.* **276**, 6098–6104.
14. Groemping, Y. & Reinstein, J. (2001). Folding properties of the nucleotide exchange factor GrpE from *Thermus thermophilus*: GrpE is a thermosensor that mediates heat shock response. *J. Mol. Biol.* **314**, 167–178.
15. Moro, F. & Muga, A. (2006). Thermal adaptation of the yeast mitochondrial Hsp70 system is regulated by the reversible unfolding of its nucleotide exchange factor. *J. Mol. Biol.* **358**, 1367–1377.
16. Mally, A. & Witt, S. N. (2001). GrpE accelerates peptide binding and release from the high affinity state of DnaK. *Nat. Struct. Biol.* **8**, 254–257.
17. Han, W. & Christen, P. (2003). Interdomain communication in the molecular chaperone DnaK. *Biochem. J.* **369**, 627–634.
18. Mehl, A. F., Heskett, L. D. & Neal, K. M. (2001). A GrpE mutant containing the NH(2)-terminal "tail" region is able to displace bound polypeptide substrate from DnaK. *Biochem. Biophys. Res. Commun.* **282**, 562–569.
19. Brehmer, D., Gässler, C., Rist, W., Mayer, M. P. & Bukau, B. (2004). Influence of GrpE on DnaK–substrate interactions. *J. Biol. Chem.* **279**, 27957–27964.
20. Chesnokova, L. S., Slepenkov, S. V., Protasevich, I. I., Sehorn, M. G., Brouillette, C. G. & Witt, S. N. (2003). Deletion of DnaK's lid strengthens binding to the nucleotide exchange factor. GrpE: a kinetic and thermodynamic analysis. *Biochemistry*, **42**, 9028–9040.
21. Gelinas, A. D., Toth, J., Bethoney, K. A., Stafford, W. F. & Harrison, C. J. (2004). Mutational analysis of the energetics of the GrpE.DnaK binding interface: equilibrium association constants by sedimentation velocity analytical ultracentrifugation. *J. Mol. Biol.* **339**, 447–458.
22. Fukada, H. & Takahashi, K. (1998). Enthalpy and heat capacity changes for the proton dissociation of various

buffer components in 0.1 M potassium chloride. *Proteins*, **33**, 159–166.
23. Gelinas, A. D., Langsetmo, K., Toth, J., Bethoney, K. A., Stafford, W. F. & Harrison, C. J. (2002). A structure-based interpretation of *E. coli* GrpE thermodynamic properties. *J. Mol. Biol.* **323**, 131–142.
24. Moro, F., Fernández-Sáiz, V. & Muga, A. (2004). The lid subdomain of DnaK is required for the stabilization of the substrate-binding site. *J. Biol. Chem.* **279**, 19600–19606.
25. Murphy, K. P. & Freire, E. (1992). Thermodynamics of structural stability and cooperative folding behaviour in proteins. *Adv. Protein Chem.* **43**, 313–361.
26. Murphy, K. P., Xie, D., Thompson, K. S., Amzel, L. M. & Freire, E. (1990). Entropy in biological binding processes: estimation of translational entropy loss. *Science*, **247**, 559–561.
27. Luque, I. & Freire, E. (1998). Structure-based prediction of binding affinities and molecular design of peptide ligands. *Methods Enzymol.* **295**, 100–127.
28. Karzai, A. W. & McMacken, R. (1996). A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. *J. Biol. Chem.* **271**, 11236–11246.
29. Montgomery, D., Jordan, R., McMacken, R. & Freire, E. (1993). Thermodynamic and structural analysis of the folding/unfolding transitions of the *Escherichia coli* molecular chaperone DnaK. *J. Mol. Biol.* **232**, 680–692.
30. Neidhart, F. C. & VanBogelen, R. A. (1987). In *Escherichia coli and Salmonella typhimurium* (Neidherdt, F. C., ed), pp. 1334–1345, American Society for Microbiology, Washington, DC.
31. Gelinas, A. D., Toth, J., Bethoney, K. A., Langsetmo, K., Stafford, W. F. & Harrison, C. J. (2003). Thermodynamic linkage in the GrpE nucleotide exchange factor, a molecular thermosensor. *Biochemistry*, **42**, 9050–9059.
32. Doig, A. J. & Sternberg, M. J. E. (1995). Side-chain conformational entropy in protein folding. *Protein Sci.* **4**, 2247–2251.
33. Horst, M., Oppliger, W., Feifel, B., Schatz, G. & Glick, B. S. (1996). The mitochondrial protein import motor: dissociation of mitochondrial hsp70 from its membrane anchor requires ATP binding rather than ATP hydrolysis. *Protein Sci.* **5**, 759–767.
34. Rist, W., Graf, C., Bukau, B. & Mayer, M. P. (2006). Amide hydrogen exchange reveals conformational changes in hsp70 chaperones important for allosteric regulation. *J. Biol. Chem.* **281**, 16493–16501.
35. Swain, J. F., Dinler, G., Sivendran, R., Montgomery, D. L., Stotz, M. & Gierasch, L. M. (2007). Hsp70 chaperone ligands control domain association *via* an allosteric mechanism mediated by the interdomain linker. *Mol. Cell*, **26**, 27–39.
36. Moro, F., Fernández, V. & Muga, A. (2003). Interdomain interaction through helices A and B of DnaK peptide binding domain. *FEBS Lett.* **533**, 119–123.
37. Drees, B. L., Grotkopp, E. K. & Nelson, H. C. (1997). The GCN4 leucine zipper can functionally substitute for the heat shock transcription factor's trimerization domain. *J. Mol. Biol.* **273**, 61–74.
38. Weissenhorn, W., Calder, L. J., Dessen, A., Laue, T., Skehel, J. J. & Wiley, D. C. (1997). Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **94**, 6065–6069.
39. Grimshaw, J. P., Jelesarov, I., Siegenthaler, R. K. & Christen, P. (2003). Thermosensor action of GrpE. The DnaK chaperone system at heat shock temperatures. *J. Biol. Chem.* **278**, 19048–19053.
40. Buchberger, A., Schröder, H., Büttner, M., Valencia, A. & Bukau, B. (1994). A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. *Nat. Struct. Biol.* **1**, 95–101.
41. Willmund, F., Mühlhaus, T., Wojciechowska, M. & Schroda, M. (2007). The NH_2-terminal domain of the chloroplast GrpE homolog CGE1 is required for dimerization and cochaperone function in vivo. *J. Biol. Chem.* **13**, 11317–11328.