Interdomain communication in the molecular chaperone DnaK

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DnaK, a heat-shock protein 70 (Hsp70) homologue in *Escherichia coli*, possesses a single tryptophan residue in its ATPase domain. Changes in the intrinsic fluorescence of DnaK offer a simple means not only to follow the binding of ATP and of ADP plus the co-chaperone GrpE to the ATPase domain, but also to investigate the kinetics of peptide binding to the substrate-binding domain of ATP·DnaK and GrpE-liganded ADP·DnaK. Addition of ATP or of ADP plus GrpE to nucleotide-free DnaK resulted in a similar decrease in intrinsic fluorescence, indicating similar open conformations of the ATPase domain under these two conditions. Binding of peptide increased the intrinsic fluorescence of both ATP·DnaK and ADP·DnaK·GrpE and rendered their spectra similar to the spectrum of ADP·DnaK with closed conformation of the ATPase domain. These results, together with the differential kinetics of peptide binding to ADP·DnaK on the one hand, and to ATP·DnaK or ADP·DnaK·GrpE on the other, suggest that ligands for either domain, i.e. ATP or ADP plus GrpE for the ATPase domain and peptides for the substrate-binding domain, shift the conformational equilibrium of both domains of DnaK towards the open and closed forms, respectively, in a concerted and parallel manner.

Key words: ATPase, GrpE, heat-shock protein 70 (Hsp70), intrinsic fluorescence, nucleotide.

INTRODUCTION

Chaperone systems of the heat-shock protein 70 (Hsp70) family facilitate the folding of nascent and denatured proteins and prevent the formation of protein aggregates. Hsp70 chaperones consist of an N-terminal ATPase domain and a C-terminal substrate-binding domain (for comprehensive reviews, see [1]). DnaK, an Hsp70 homologue of *Escherichia coli*, binds hydrophobic peptides and segments of denatured proteins in extended conformation [2]. DnaK alternates between its ATP-liganded T state, characterized by a low affinity for target polypeptides as well as fast rates of binding and release, and the ADP-liganded R state with high affinity and low exchange rates for substrates [3–6]. In its chaperone action, DnaK co-operates with two cohort heat-shock proteins, DnaJ and GrpE [7]. The conversion of DnaK from its T state to the R state is triggered by DnaJ, which accelerates the hydrolysis of DnaK-bound ATP. GrpE, the second co-chaperone, facilitates the exchange of DnaK-bound ADP with ATP, converting DnaK back to the T state and thus completing the chaperone cycle. Unlike DnaJ, GrpE forms a stable complex with the ATPase domain of ADP-liganded or nucleotide-free DnaK [8–10].

The control of substrate binding and release by the nucleotide status of DnaK implies that conformational changes in the ATPase domain are communicated to the substrate-binding domain. Conversely, binding of peptide to ATP-liganded DnaK increases its ATPase activity, i.e. the substrate-induced conformational change extends to the ATPase domain. This two-way interdomain communication is an essential feature of the ATP-dependent chaperone cycle of DnaK and other Hsp70 chaperones [3,6,11–15].

The single tryptophan residue (Trp-102) of DnaK is located in a β-strand in the upper part of the IB subdomain which forms a lobe constituting one wall of the nucleotide-binding cleft in the ATPase domain [10]. The fluorescence of this tryptophan residue in the ATPase domain changes with the nucleotide status of DnaK [16,17] and thus may be used to monitor the $T \leftrightarrow R$ interconversion of DnaK [14,15]. Moreover, binding of peptide to ATP-liganded DnaK has been reported to increase the tryptophan fluorescence independently of ATP hydrolysis [18], suggesting that the kinetics of peptide binding to T-state DnaK might be determined by tracking changes in intrinsic fluorescence. In this study, the intrinsic fluorescence signal and the kinetics of ligand binding served to explore the conformational interdomain communication triggered by peptides interacting with the substrate-binding domain or by nucleotides and GrpE interacting with the ATPase domain.

MATERIALS AND METHODS

Materials

DnaK was purified as described previously [19]. The stock solution was stored at −80 °C in 50 mM Tris/HCl, 10 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol, pH 8.0. The nucleotide content of the preparation was less than 0.1 mol/mol of DnaK [19]. For experimentation, samples of DnaK were transferred into assay buffer (25 mM Hepes/KOH, 100 mM KCl and 10 mM $MgCl_2$, pH 7.0) with NAP-5 columns (Amersham Biosciences). The protein concentration was determined photometrically with a molar absorption coefficient, ϵ_{280}, of 14500 $M^{-1} \cdot cm^{-1}$ [20]. DnaJ and GrpE, prepared as reported previously [9,21], were gifts from H.-J. Schönfeld, F. Hoffmann-La Roche, Basel, Switzerland. The stock solutions were stored at −80 °C in 50 mM Tris/HCl, pH 7.6. The indicated concentrations of GrpE are those of its dimeric form, which were determined photometrically with a molar absorption coefficient

Abbreviations used: acrylodan, 6-acrylolyl-2-dimethylaminonaphthalene; Hsp70, heat-shock protein 70.

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of $\epsilon_{280} = 2720\ M^{-1} \cdot cm^{-1}$ (H.-J. Schönfeld, personal communication).

Peptides NR (NRLLLTG) and pp (CALLQSRLLLSAPRRAAATARA; a derivative of the prepiece of chicken mitochondrial aspartate aminotransferase) with a purity $> 90\%$ were purchased from Chiron. The concentration of their stock solutions was 1 mM in water (with 20 mM 1,4-dithiothreitol for peptide pp). Peptides were labelled with the environmentally sensitive fluorophore acrylodan (6-acrylolyl-2-dimethylaminonaphthalene; from Molecular Probes) at the α-amine group of peptide NR or the thiol group of peptide pp, and purified as described previously [5]. The ATP and ADP stock solutions (100 mM disodium salt, pH 7.0; from Fluka) in water were stored in small portions at -80 °C. [2,5′,8-^{3}H]ATP ammonium salt (41.0 Ci/mmol) was purchased from Amersham Biosciences. All experiments in this study were performed in assay buffer (25 mM Hepes/KOH, 100 mM KCl and 10 mM $MgCl_2$, pH 7.0) at 25 °C if not indicated otherwise.

Fluorescence spectra

Fluorescence spectra were recorded with a Spex Fluorolog spectrofluorimeter in a 1 cm × 1 cm cuvette. For measuring acrylodan fluoresence, the excitation wavelength was set at 370 nm, and the bandpasses of both excitation and emission were 4.6 nm. For measuring intrinsic fluorescence of DnaK, the excitation wavelength was set at 290 nm, and the excitation and emission bandpasses were 18.5 nm and 9.2 nm respectively.

Fast kinetic measurements

An SX18 MV stopped-flow machine from Applied Photophysics served to record the changes in intrinsic fluorescence of DnaK (excitation at 290 nm, bandpass 15 nm, emission high-pass filter 320 nm) and the changes in fluorescence of acrylodan-labelled peptides (excitation at 370 nm, bandpass 12 nm, emission high-pass filter 455 nm). All experiments were performed in assay buffer at 25 °C and were started by mixing equal volumes ($\approx 70\ \mu$l each) of the two reaction solutions. All traces were obtained by averaging at least nine measurements. The reaction traces were fitted with single-exponential functions with the software provided by the manufacturer of the instrument, if not indicated otherwise.

ATPase assays

ATPase activity of DnaK was measured under single-turnover conditions as described in [14]. The data were analysed with Sigmaplot 5.0.

RESULTS

Binding of peptides to ATP · DnaK complex increases intrinsic fluorescence

Consistent with a recent report [18], addition of either peptide pp or NR to ATP-liganded DnaK triggered a rapid increase in tryptophan fluorescence (Figure 1A). The increase in intrinsic fluorescence triggered by peptide binding does not signal the hydrolysis of ATP, since the rate of ATP hydrolysis, determined by single-turnover assays in the presence of peptides pp or NR, is four orders of magnitude slower than the rate of the fluorescence change (Table 1). The plots of the k_{obs} values of complex formation versus the concentrations of peptide pp and NR were linear (Figure 1B). It has to be noted that the dissociation equilibrium constants K_d for peptides pp and NR were 4.7 and 54 μM, respectively (Table 2). Thus in the case of pp the highest concentration used (100 μM) ensures virtual saturation. The on (k_{+1}) and off (k_{-1}) rate constants obtained from the slope and intercept, respectively, proved to be similar to the rate constants derived from measurements with acrylodan-labelled peptides pp and NR (Table 2). The difference in the amplitudes of the fluorescence changes induced by the two peptides (Figure 1A) reflects the difference in their binding affinities for DnaK (for K_d values see Table 2). As reported elsewhere [18], addition of peptides to ADP · DnaK did not change the intrinsic fluorescence (results not shown).

The fluorescence spectra of nucleotide-free DnaK and ADP · DnaK are virtually the same (results not shown). In agreement with previous reports [4,16,17], the intrinsic fluorescence of ATP · DnaK, however, was found to be considerably lower than that of nucleotide-free DnaK or ADP · DnaK, with the emission maximum shifted from 351 to 345 nm. On binding of peptide pp to ATP · DnaK, the emission maximum of tryptophan fluorescence shifted back to 351 nm and the fluorescence intensity increased back to the same level as that of nucleotide-free DnaK or ADP · DnaK (Figure 1C). The increase in intrinsic fluorescence at 351 nm upon addition of peptide to ATP · DnaK may be used not only for kinetic measurements (Figure 1B) but also for determining the dissociation equilibrium constant by titration (Figure 1D). The K_d values calculated from the kinetic data (Figure 1B) and those determined by titration agree very well (Table 2).

Table 1 Comparison of the rate of ATP hydrolysis by DnaK in the presence of peptides and the rate of complex formation of ATP · DnaK with peptide

For determination of the k'_{cat} value for single-turnover ATPase activity, the peptides were added to the preformed ATP · DnaK complex. The final concentration of ATP · DnaK complex was 1 μM and that of both peptides was 50 μM. To measure the rate of complex formation (k_{obs}), the reactions were started by mixing 1 μM DnaK/5 mM ATP with 50 μM peptides/5 mM ATP in the stopped-flow apparatus at 25 °C. The concentrations indicated are those after mixing. For details see the Materials and methods section. Reported values are means ± S.D. from three independent experiments.

	ATPase activity (k'_{cat}; s^{-1})	Complex formation (k_{obs}; s^{-1})
No peptide	0.0003 ± 0.0002	–
NR	0.0052 ± 0.0014	36 ± 2.3
pp	0.0089 ± 0.0019	57 ± 2.8

Table 2 Rate constants and dissociation equilibrium constants of ATP · DnaK for peptides pp and NR

The values of k_{+1} and k_{-1} were calculated from the data of Figure 1(B). Reported values are means (± S.D.) from three independent experiments. a-pp, Acrylodan-labelled peptide pp; a-NR, acrylodan-labelled peptide NR.

Peptide	k_{+1} ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	K_d (μM)
pp	998 500	4.7	4.7† (4.1)‡
a-pp	450 000*	1.8*	4.2† (2.2 ± 0.8)§
NR	328 000	17.9	54†
a-NR	104 000*	4.5*	43† (32 ± 2.3)§

* From [26].
† Calculated from k_{-1} and k_{+1}.
‡ Determined by titration (Figure 1D).
§ Determined by titrating 50 nM acrylodan-labelled peptides in assay buffer containing 1 mM ATP with increasing concentrations of DnaK (results not shown).

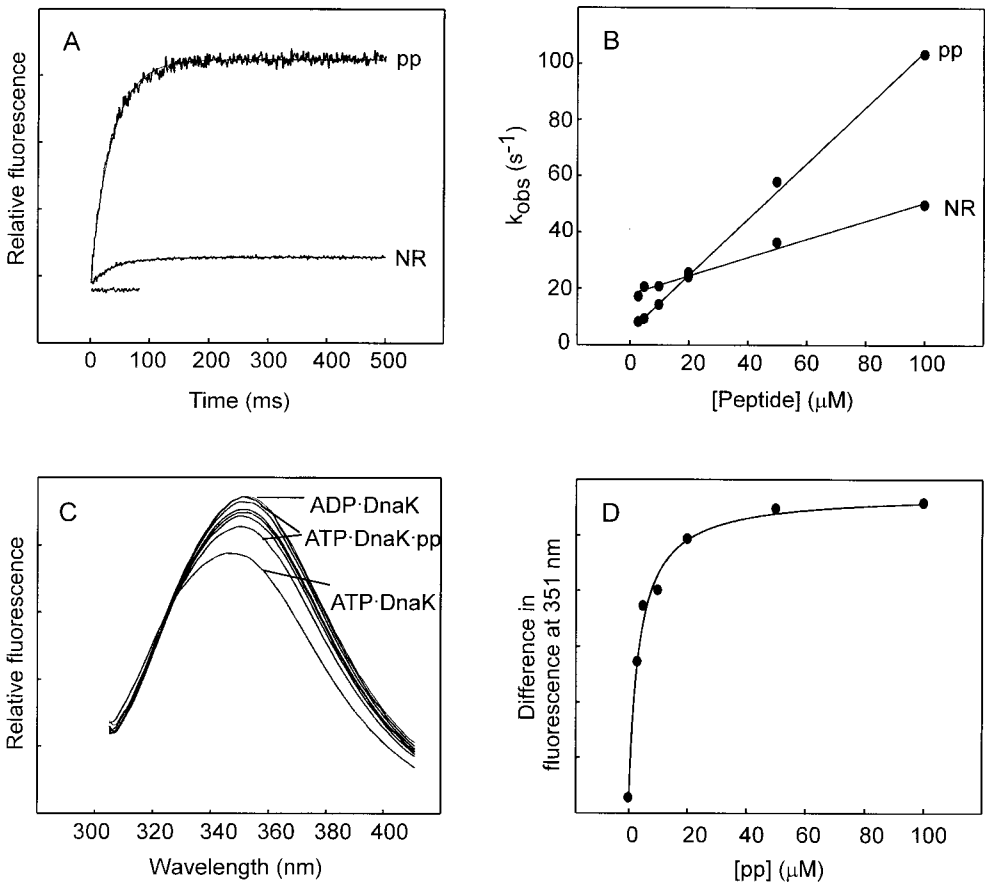


Figure 1 Kinetics of peptide binding to ATP · DnaK followed by intrinsic fluorescence

(**A**) The rates of complex formation between peptides and ATP · DnaK were determined by monitoring the increase in intrinsic fluorescence with a stopped-flow machine (for details see the Materials and methods section). The reaction was started by mixing 1 μM DnaK/5 mM ATP with 20 μM peptide/5 mM ATP. The concentrations indicated are those after mixing. The solution in the first syringe was equilibrated for at least 2 min before the experiments. The pseudo-first-order rates k_{obs} with peptide pp and NR were 24 ± 1.7 s^{-1} and 20 ± 1.4 s^{-1}, respectively. Under all conditions, the reaction traces started at the baseline (horizontal line) of [DnaK + ATP] + [buffer]. (**B**) The values of k_{obs} of complex formation of ATP · DnaK with peptides pp and NR are plotted as a function of peptide concentration. The rates were measured in experiments as shown in (**A**). (**C**) Peptide binding to ATP · DnaK increases the intrinsic fluorescence. DnaK (1 μM) in assay buffer containing 1 mM ATP was titrated with increasing concentrations of peptide pp. Emission spectra were recorded after the reactions had reached equilibrium (see **A**). (**D**) The titration curve was derived from the data of (**C**) by plotting the differences in fluorescence at 351 nm against the total peptide concentration. The data points were fitted with the following non-linear regression equation: $\Delta F = (\Delta F_{max}/2 \times L_t) \times \{(L_t + P_t + K_d) - [(L_t + P_t + K_d)^2 - (4 \times L_t \times P_t)]^{0.5}\}$, where ΔF is the change in relative fluorescence, L_t is the total concentration of DnaK, and P_t is the total concentration of peptide pp. The calculated value of K_d is 4.1 ± 0.5 μM.

Interaction of GrpE with ADP · DnaK decreases intrinsic fluorescence

Dimeric GrpE forms a stable complex with nucleotide-free DnaK or ADP · DnaK [8–10]. The effects of binding of GrpE on the conformation of the ATPase domain of DnaK in the presence and absence of ADP were again monitored by the fluorescence of the single tryptophan residue of DnaK. GrpE contains no tryptophan and only one tyrosine residue and therefore produces a minimal fluorescence signal when excited at 290 nm. Similar to the effect of ATP on DnaK, GrpE shifts the emission maximum from 351 to 347 nm and decreases the intrinsic fluorescence of ADP · DnaK, although to a lesser extent even at saturating concentrations (Figure 2A). The reaction trace was fitted with a single-exponential function, yielding the apparent rate constant for the formation of the ADP · DnaK · GrpE complex (Figure 2B). In a control experiment, addition of ADP to the preformed DnaK · GrpE complex brought about a similar fluorescence decrease (Figures 2C and 2D), indicating that the change in intrinsic fluorescence reflects a new conformational equilibrium of DnaK induced by a synergistic effect of GrpE and ADP. With our preparation of nucleotide-free DnaK, we observed only a small decrease after the addition of GrpE (Figure 2B), which seems to be due to contaminating traces of ADP [19]. This interpretation is consistent with a previous report that addition of GrpE does not cause any observable change in the intrinsic fluorescence signal of nucleotide-free DnaK [22]. Addition of GrpE to ATP-liganded DnaK did not elicit any observable fluorescence signal (results not shown).

Binding of peptide to GrpE-liganded ADP · DnaK

The binding of peptide pp to preformed ADP · DnaK · GrpE was again monitored by changes in intrinsic fluorescence. Addition of peptide pp increased the intrinsic fluorescence (Figure 3A) as observed when adding peptide to ATP · DnaK (Figure 1C). The reaction trace for peptide binding was fitted with a double-exponential function (Figure 3B). The rate of the first phase is

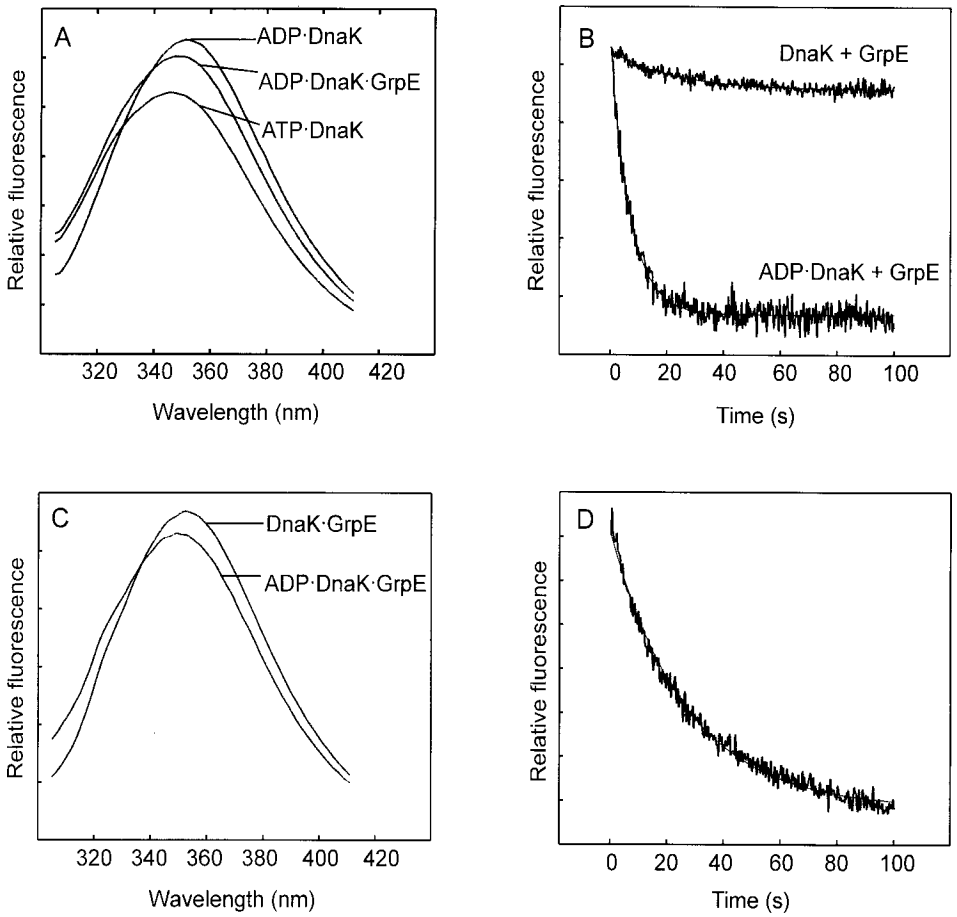


Figure 2 GrpE decreases the intrinsic fluorescence of ADP · DnaK

(**A**) The spectra of ADP · DnaK and ATP · DnaK were compared with that of ADP · DnaK · GrpE. The concentrations of DnaK, nucleotides and GrpE were 1 μM, 1 mM and 2 μM respectively. The K_d value of the ADP · DnaK · GrpE complex is 0.22 μM [22]; the 2 μM concentration of GrpE dimer thus ensures saturation. The spectra were recorded after the reactions had reached steady state or equilibrium. (**B**) The rates of complex formation with GrpE were determined by monitoring the decrease in intrinsic fluorescence with a stopped-flow apparatus. The reactions were started by mixing 1 μM DnaK with or without 1 mM ADP or with 2 μM GrpE with or without 1 mM ADP. The value of k_{obs} was 0.11 ± 0.03 s^{-1} for ADP-liganded DnaK. (**C**) DnaK (1 μM) in assay buffer was incubated with 2 μM GrpE for 10 min to form the DnaK · GrpE complex. Then ADP was added to a final concentration of 1 mM. The spectrum was recorded after the reaction had reached equilibrium and was corrected for the dilution factor. (**D**) The reaction was started by mixing 1 μM DnaK/2 μM GrpE with 1 mM ADP in a stopped-flow apparatus and monitored by the change in the intrinsic fluorescence of DnaK ($k_{obs} = 0.06 \pm 0.01$ s^{-1}). All concentrations indicated are those after mixing.

comparable with that of complex formation of peptide pp with ATP · DnaK under the same conditions (Figure 1A). With nucleotide-free DnaK · GrpE, no signal change was observed after mixing with peptide pp (Figure 3B), confirming that ADP-liganded DnaK · GrpE indeed differs in conformation from nucleotide-free DnaK · GrpE. By titrating ADP · DnaK · GrpE with peptide pp, we determined the dissociation equilibrium constant K_d of peptide pp to be 0.4 μM (Figure 3C).

The similarity of the effect of GrpE on ADP · DnaK with that of ATP on nucleotide-free DnaK is consistent in part with a recent report that GrpE, similar to ATP, accelerates peptide binding to and release from DnaK [23]. Indeed, in the presence of GrpE, both the binding of acrylodan-labelled peptide pp to and its release from ADP · DnaK (Figures 4A and 4B, respectively) were about one order of magnitude faster than in the absence of GrpE (Figures 4C and 4D). However, in contrast to the previous report [23], peptide binding to and release from nucleotide-free DnaK were not accelerated by the addition of GrpE (Figure 4C and 4D). Apparently, the acceleration of peptide binding and release by GrpE depends on the presence of ADP, corroborating the notion of a synergistic effect of ADP and GrpE as observed with the decrease in intrinsic fluorescence (Figure 2).

DISCUSSION

In this study, we explored the conformational change in the ATPase domain of ATP-liganded DnaK upon binding of peptide to its substrate-binding domain [18] with the well-characterized peptides pp and NR [2,14]. The fluorescence intensity and the wavelength of maximum emission of the ATP · DnaK · peptide complex were similar to those of ADP · DnaK (Figure 1C). In accordance with this finding, no fluorescence change was observed upon addition of DnaJ, which stimulates the ATPase activity of DnaK, to ATP · DnaK · peptide (results not shown), suggesting that the conformations of ATP · DnaK · peptide and ADP · DnaK · peptide, at least in the ATPase domain, are similar. This notion is supported by the previous observation that binding of peptide to ATP · DnaK changes the pattern of trypsin digestion to that of ATP · DnaK after addition of DnaJ, i.e. of ADP · DnaK

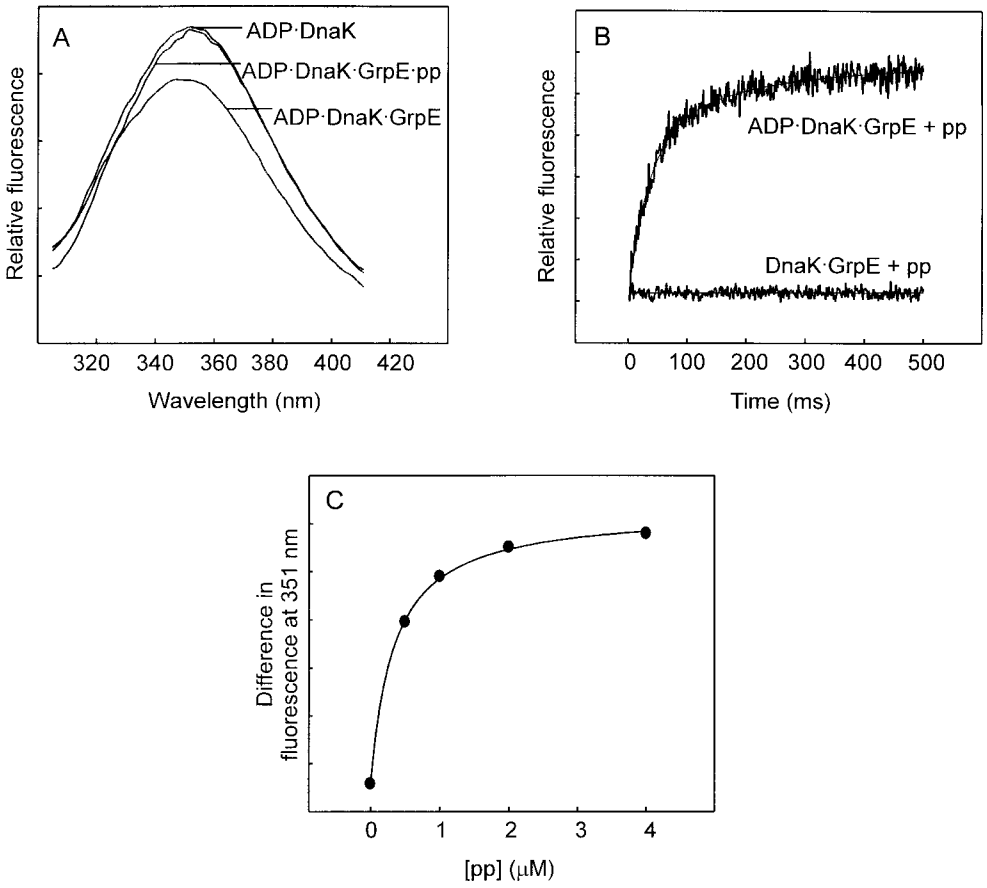


Figure 3 Peptide binding increases the intrinsic fluorescence of DnaK · GrpE

(**A**) The fluorescence spectra of ADP · DnaK, ADP · DnaK · GrpE were compared with that of ADP · DnaK · GrpE · pp. The concentrations of DnaK, GrpE, ADP and peptide pp were 1 μM, 2 μM, 1 mM and 20 μM respectively. (**B**) The rate of peptide binding was determined by monitoring the intrinsic fluorescence change with a stopped-flow device. The reaction was triggered by mixing 1 μM DnaK/2 μM GrpE with or without 1 mM ADP with 20 μM peptide pp with or without 1 mM ADP. The solution in the first syringe was equilibrated for 2 min to allow complex formation. The traces were fitted with a double-exponential function with $k_{obs1} = 21 \pm 3\ s^{-1}$ (amplitude 76% of total) and $k_{obs2} = 5.7 \pm 1.4\ s^{-1}$. (**C**) DnaK (1 μM) in assay buffer containing 1 mM ADP was first incubated with 2 μM GrpE for 2 min to form the DnaK · GrpE complex. The complex was then titrated with 0.5–4 μM peptide pp. The titration curve was analysed as described in the legend of Figure 1(D). The calculated value of K_d is 0.4 ± 0.08 μM. The final concentrations are indicated.

[24]. The change in the intrinsic fluorescence signal upon peptide binding is thus a structural correlate of the interdomain communication in DnaK [4–6,11], for which as yet only functional evidence, i.e. the stimulation of the ATPase activity by peptide [6,25], has been provided.

According to the linear concentration dependence of the k_{obs} of peptide binding (Figure 1B), complex formation, as signalled by intrinsic fluorescence, is a one-step process. Assuming that the complex forms according to:

$$\mathrm{ATP \cdot DnaK + P} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{ATP \cdot DnaK^{*} + P} \overset{k_{+2}}{\rightleftarrows} \mathrm{ATP \cdot DnaK^{*} \cdot P} \qquad (1)$$

where DnaK* denotes the conformer with high fluorescence intensity, ATP · DnaK is the predominant conformer in equilibrium 1 and the binding of the peptide (P) is much faster than the conformational changes in the peptide-binding domain and ATPase domain, which precede the binding of the peptide and underlie the observed fluorescence signal. This interpretation of the kinetics of peptide binding also agrees with a previous report [18] that peptide binding to T-state DnaK is limited by the open/closed equilibrium of the peptide-binding site.

The peptide-induced increase in intrinsic fluorescence provides a simple means to determine kinetic and thermodynamic parameters of the interaction of ATP · DnaK with unlabelled, instead of fluorescence-labelled, substrates. The rates of the change in intrinsic fluorescence upon binding of unlabelled peptides correspond well with the rates measured with acrylodan-labelled peptides (Table 2). The agreement not only verifies previous kinetic data obtained with acrylodan-labelled peptides [5,26] but also indicates that the shifts of the conformational equilibrium 1 (ATP · DnaK ↔ ATP · DnaK*; see eqn 1), which occur both in the substrate-binding domain and in the ATPase domain, are concerted processes. In agreement with previous reports that fluorescence-labelled *retro inverso* peptides (peptides of D-amino acids with, as compared with the reference L-peptides, reverse sequence and thus exhibiting in extended conformation the same side-chain topology as the reference L-peptides) do not bind to DnaK [27], unlabelled *retro inverso* peptides did not trigger changes in the intrinsic fluorescence (results not shown).

We found that binding of GrpE to DnaK causes a similar blue shift in λ_{max}^{em} and a similar decrease in fluorescence as the binding of ATP and that this change in intrinsic fluorescence depends on the simultaneous presence of ADP (Figures 2A and 2B). By the

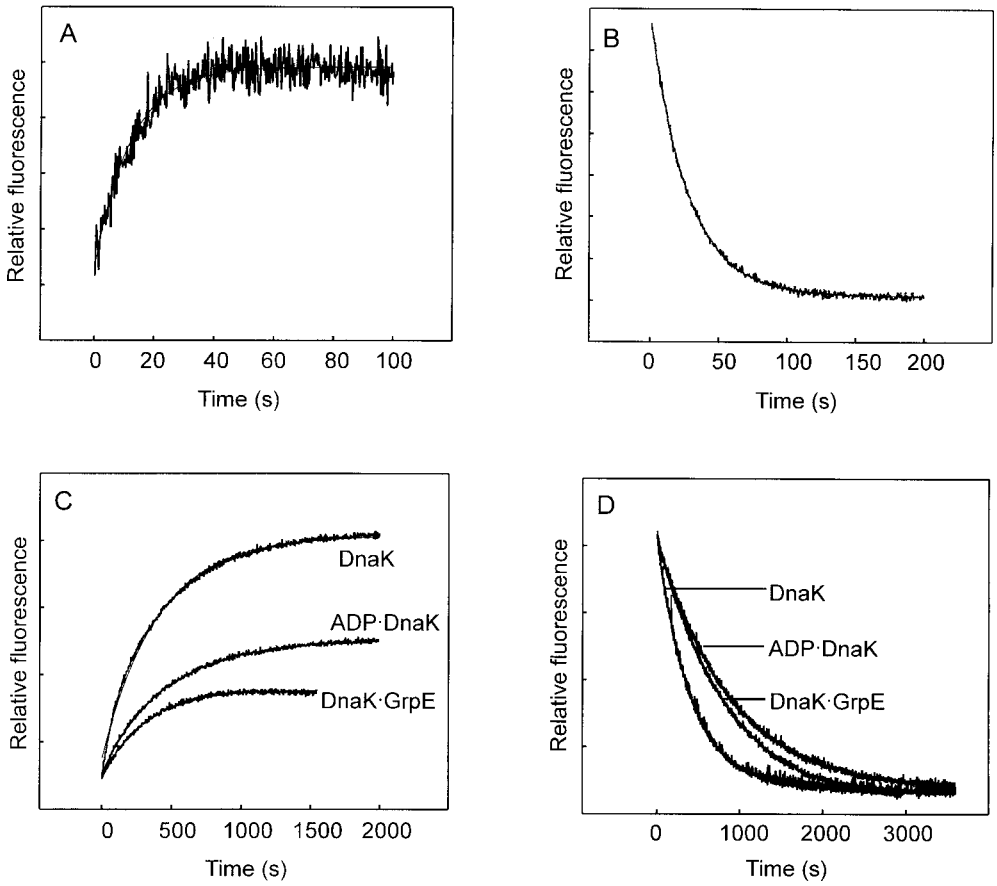


Figure 4 GrpE-accelerated peptide binding to and release from ADP · DnaK

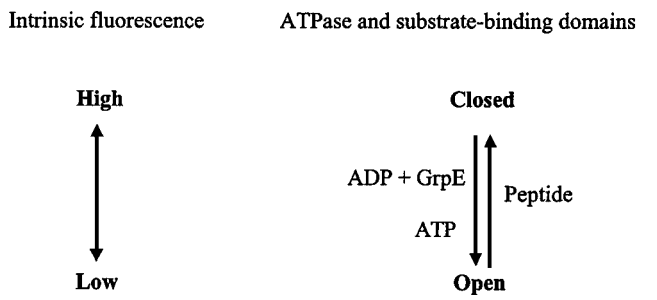
(**A**) The rate of binding of acrylodan-labelled peptide pp (a-pp) to ADP · DnaK · GrpE was measured by monitoring the increase in fluorescence with the stopped-flow device. The reaction ($k_{obs} = 0.08 \pm 0.01$ s^{-1}) was started by mixing 1 μM DnaK/2 μM GrpE/1 mM ADP with 50 nM a-pp/1 mM ADP. The solution in the first syringe was incubated for 10 min to form the complex. (**B**) The release of a-pp from ADP · DnaK · a-pp ($k_{off} = 0.04 \pm 0.02$ s^{-1}) was triggered by mixing 1 μM DnaK/50 nM a-pp/1 mM ADP with 2 μM GrpE/20 μM unlabelled peptide pp/1 mM ADP. The solution in the first syringe was pre-incubated for 90 min to form the complex. (**C**) The binding of peptides to the indicated conformers of DnaK was monitored by the increase in acrylodan fluorescence. Either DnaK (1 μM) was added to 50 nM a-pp in assay buffer without ($k_{obs} = 0.007 \pm 0.002$ s^{-1}) or with 1 mM ADP ($k_{obs} = 0.006 \pm 0.002$ s^{-1}), or DnaK · GrpE complex, preformed by incubating 1 μM DnaK and 2 μM GrpE for 10 min, was added to 50 nM a-pp in assay buffer without ADP ($k_{obs} = 0.008 \pm 0.002$ s^{-1}). (**D**) To follow the release of peptide from the indicated conformers of DnaK, unlabelled peptide pp (20 μM) was added to the conformers, which were formed by incubating 1 μM DnaK and 50 nM a-pp for 90 min in buffer containing either 1 mM ADP ($k_{off} = 0.001 \pm 0.0003$ s^{-1}) or 2 μM GrpE ($k_{off} = 0.001 \pm 0.0004$ s^{-1}), or no such addition ($k_{off} = 0.002 \pm 0.0004$ s^{-1}).

Table 3 Effects of nucleotides and GrpE on the intrinsic fluoresence and on peptide binding to and release from Dnak

Fluorescence intensity data were derived from Figures 1(C) and 2(A); the intrinsic fluorescence intensity of ADP · DnaK was set at 100%. The pseudo-first-order rate constants (k_{obs}) were determined with a stopped-flow device by mixing 50 nM acrylodan-labelled peptide pp with 1 μM DnaK in assay buffer containing 1 mM ADP and 1 mM ATP (results not shown). k_{on} data were calculated from k_{obs} and k_{off}. k_{off} data were determined by monitoring the acrylodan fluorescence after mixing 1 μM DnaK/50 nM acrylodan-labelled peptide pp with 20 μM unlabelled pp with a stopped-flow device. For ADP-state measurements, 1 mM ADP was in both syringes and the solution in the first syringe was equilibrated for 90 min before mixing. For ATP-state measurements, 1 mM ATP was in both syringes and the solution in the first syringe was equilibrated for 2 min before mixing. K_d was determined by titrating 50 nM acrylodan-labelled pp with DnaK in assay buffer containing 1 mM ADP or ATP. The concentrations indicated are those after mixing. Reported values are means ± S.D. from three independent experiments.

	Fluorescence intensity (%)	λ_{max}^{em} (nm)	Rates of binding and release of pp			K_d for pp (μM)
			k_{obs} (s^{-1})	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	
ADP · DnaK	100	351	0.008 ± 0.002	6000	0.002 ± 0.001	0.07 ± 0.02
ADP · DnaK · GrpE	89	347	0.08 ± 0.01*	40000	0.04 ± 0.02†	0.4 ± 0.08‡
ATP · DnaK	77	345	2.5 ± 0.5	470000	2.1 ± 0.6	2.2 ± 0.3

* Taken from Figure 4(A).
† Taken from Figure 4(B).
‡ Taken from Figure 3(C).



Scheme 1 Model of the conformational coupling of the ATPase and substrate-binding domains of DnaK

Both domains exist in equilibrium between an open and a closed conformation. Ligands for the two domains, i.e. ADP, ATP and GrpE for the ATPase domain, and peptides for the substrate-binding domain, shift the conformational equilibrium in a concerted and parallel manner for both domains. The intrinsic fluorescence is mainly due to the single tryptophan residue located in the ATPase domain.

criterion of the intrinsic fluorescence spectrum, the conformation of the ATPase domain of ADP·DnaK·GrpE is an intermediary between the conformations of ADP·DnaK and ATP·DnaK. The increase in intrinsic fluorescence upon binding of peptide to DnaK·GrpE also depends on the presence of ADP (Figure 3B). GrpE accelerates peptide binding to and release from ADP·DnaK. This effect is not observed with nucleotide-free DnaK (Figure 4); apparently, it also depends on the simultaneous presence of ADP and GrpE. However, our finding that the effects of GrpE depend on the simultaneous presence of ADP is at variance with a previous report that the GrpE-facilitated binding of peptide to and release from DnaK is due only to the binding of GrpE to DnaK and independent of the presence of ADP [23]. The reason for the discrepancy is unclear. The effect of GrpE on peptide binding and release in the presence of ADP does not seem to be of physiological significance, because ATP is two orders of magnitude more effective than GrpE in accelerating peptide binding and release (Table 3). This argument is all the more valid as the intracellular concentration of GrpE dimer is only one-sixth of that of DnaK [28].

The similar changes in the intrinsic fluorescence spectra suggest that the conformational equilibrium of the ATPase domain in ADP·DnaK·GrpE is shifted in the same direction as in ATP·DnaK (Figure 2A). The dissociation rate constant of ATP·DnaK ($k_{off} = 15\ s^{-1}$) is considerably faster compared with that of ADP·DnaK ($k_{off} = 0.022\ s^{-1}$) [29]. In the presence of GrpE, the rate constant of ADP dissociation, determined by displacing ADP with ATP, was in the range of 1–5 s^{-1} when measured using various procedures [15]. Binding of GrpE to ADP·DnaK opens the nucleotide-binding cleft [10] and decreases the intrinsic fluorescence (Figure 2A). The nucleotide-binding cleft is closed in the ADP state with slow nucleotide dissociation and high intrinsic fluorescence, while the nucleotide-binding cleft is open in the ATP state with fast nucleotide dissociation and low intrinsic fluorescence. Thus the conformation of the ATPase domain of ADP·DnaK·GrpE corresponds, by the criteria of not only intrinsic fluorescence but also the nucleotide dissociation rate constant, to a state that is intermediate between the ADP- and the ATP-liganded states, i.e. of the two successive stages of the chaperone cycle that are interconverted by the co-chaperone GrpE. The effects of nucleotides and GrpE on the conformation of the ATPase domain correlate well with their effects on peptide binding and release, i.e. on the conformation of the substrate-binding domain (for a synopsis, see Table 3). The similarity of the intrinsic fluorescence spectra of ATP·DnaK·peptide and ADP·DnaK·GrpE·peptide with that of ADP·DnaK (Figures 1C and 3A), respectively, suggests that the conformations of the ATPase domain in these three conformers are similar, i.e. in a closed state. A recent report indicated that peptide release from ATP·DnaK·peptide involves a conformational shift of the substrate-binding domain (including both β-subdomain and α-helical lid subdomain) to the open state [30], implying that the binding of peptide shifts the conformational equilibrium of ATP·DnaK towards the closed state. Because a given peptide interacts with the same residues and in a similar way in either conformer of DnaK [31], it seems that peptides shift the conformational equilibrium of the substrate-binding domain in general in the direction towards the closed state. This conclusion is consistent with the previous observation that the substrate-binding domain assumes a more structured conformation when liganded with a peptide [32]. Apparently, this open → closed conformational change is transferred to the ATPase domain where it induces a parallel change as indicated by changes in the fluorescence spectrum brought about by binding of peptide (Figures 1C and 3A).

On the basis of the structural and functional parameters, we propose a model for the conformational equilibrium of the two domains of DnaK and its modulation by various ligands (Scheme 1). Apparently, both the ATPase and substrate-binding domain of DnaK exist in an equilibrium between 'closed' and 'open' states. The shift from one state to the other is induced by ligands of either domain and occurs in both domains in a parallel manner. With the concerted shift of both domains from the closed state to the open state, both nucleotide dissociation [29] and substrate binding and release are accelerated (Table 2). The conformational change in the substrate-binding domain includes the β-subdomain and α-helix lid subdomain [2,30–32]. GrpE binds to the ATPase domain of ADP·DnaK and opens the nucleotide-binding cleft [10]. The shift towards the open state of the substrate-binding domain in GrpE-liganded ADP·DnaK appears to be less extensive than that in ATP-liganded DnaK and might be largely limited to the β-subdomain [23].

In summary, our study suggests that the interdomain communication of DnaK involves concerted and parallel shifts of the open–closed conformational equilibrium of both domains that are controlled by ligands of both domains. ATP and ADP plus GrpE shift the conformations of both domains from the closed state to the open state, characterized by fast nucleotide dissociation and fast peptide binding and release; peptides, by binding to the substrate-binding domain, shift the equilibrium toward the closed conformation of DnaK with slow nucleotide dissociation and slow rates of peptide binding and release. The change in intrinsic fluorescence of DnaK reflects the shift of the conformational equilibrium from one state to the other.

REFERENCES

1 Frydman, J. (2001) Folding of newly translated proteins *in vivo*: the role of molecular chaperones. Annu. Rev. Biochem. **70**, 603–647
2 Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E. and Hendrickson, W. A. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science **272**, 1606–1614
3 Flynn, G. C., Chappell, T. G. and Rothman, J. E. (1989) Peptide binding and release by proteins implicated as catalysts of protein assembly. Science **245**, 385–390
4 Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J. and Fink, A. L. (1993) ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. Nature (London) **365**, 664–666
5 Schmid, D., Baici, A., Gehring, H. and Christen, P. (1994) Kinetics of molecular chaperone action. Science **263**, 971–973
6 McCarty, J. S., Buchberger, A., Reinstein, J. and Bukau, B. (1995) The role of ATP in the functional cycle of the DnaK chaperone system. J. Mol. Biol. **249**, 126–137

7 Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. U.S.A. **88**, 2874–2878
8 Buchberger, A., Schröder, H., Büttner, M., Valencia, A. and 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
9 Schönfeld, H.-J., Schmidt, D., Schröder, H. and Bukau, B. (1995) The DnaK chaperone system of *Escherichia coli*: quaternary structures and interactions of the DnaK and GrpE components. J. Biol. Chem. **270**, 2183–2189
10 Harrison, C. J., Hayer-Hartl, M., Liberto, M. D., Hartl, F.-U. and Kuriyan, J. (1997) Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. Nature (London) **276**, 431–435
11 Ziegelhoffer, T., Lopez-Buesa, P. and Craig, E. (1995) The dissociation of ATP from hsp70 of *Saccharomyces cerevisiae* is stimulated by both Ydj1p and peptide substrates. J. Biol. Chem. **270**, 10412–10419
12 Karzai, A. W. and McMacken, R. (1996) A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. J. Biol. Chem. **271**, 11236–11246
13 Pierpaoli, E. V., Sandmeier, E., Baici, A., Schönfeld, H.-J., Gisler, S. and Christen, P. (1997) The power stroke of the DnaK/DnaJ/GrpE molecular chaperone system. J. Mol. Biol. **269**, 757–768
14 Pierpaoli, E. V., Sandmeier, E., Schönfeld, H.-J. and Christen, P. (1998) Control of the DnaK chaperone cycle by substoichiometric concentrations of the co-chaperones DnaJ and GrpE. J. Biol. Chem. **273**, 6643–6649
15 Grimshaw, J. P., Jelesarov, I., Schönfeld, H.-J. and Christen, P. (2001) Reversible thermal transition in GrpE, the nucleotide exchange factor of the DnaK heat-shock system. J. Biol. Chem. **276**, 2098–2104
16 Palleros, D. R., Reid, K. L., McCarty, J. S., Walker, G. C. and Fink, A. L. (1992) DnaK, hsp73, and their molten globules. Two different ways heat shock proteins respond to heat. J. Biol. Chem. **267**, 5279–5285
17 Banecki, B., Zylicz, M., Bertoli, E. and Tanfani, F. (1992) Structural and functional relationships in DnaK and DnaK756 heat-shock proteins from *Escherichia coli*. J. Biol. Chem. **267**, 25051–25058
18 Slepenkov, S. V. and Witt, S. N. (1998) Kinetics of the reactions of the *Escherichia coli* molecular chaperone DnaK with ATP: evidence that a three-step reaction precedes ATP hydrolysis. Biochemistry **37**, 16749–16756
19 Feifel, B., Sandmeier, E., Schönfeld, H.-J. and Christen, P. (1996) Potassium ions and the molecular-chaperone activity of DnaK. Eur. J. Biochem. **237**, 318–321
20 Hellebust, H., Uhlen, M. and Enfors, S. O. (1990) Interaction between heat shock protein DnaK and recombinant staphylococcal protein A. J. Bacteriol. **172**, 5030–5034
21 Schönfeld, H.-J., Schmidt, D. and Zulauf, M. (1995) Investigation of the molecular chaperone DnaJ by analytical ultracentrifugation. Progr. Colloid Polym. Sci. **99**, 7–10
22 Packschies, L., Theyssen, H., Buchberger, A., Bukau, B., Goody, R. S. and Reinstein, J. (1997) GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. Biochemistry **36**, 3417–3422
23 Mally, A. and Witt, S. N. (2001) GrpE accelerates peptide binding and release from the high affinity state of DnaK. Nat. Struct. Biol. **8**, 254–257
24 Buchberger, A., Theyssen, H., Schröder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J. and Bukau, B. (1995) Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. J. Biol. Chem. **270**, 16903–16910
25 Jordan, R. and McMacken, R. (1995) Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. J. Biol. Chem. **270**, 4563–4569
26 Pierpaoli, E. V., Gisler, S. M. and Christen, P. (1998) Sequence-specific rates of interaction of target peptides with the molecular chaperones DnaK and DnaJ. Biochemistry **37**, 16741–16748
27 Feifel, B., Schönfeld, H.-J. and Christen, P. (1998) D-peptide ligands for the co-chaperone DnaJ. J. Biol. Chem. **273**, 11999–12002
28 Neidhardt, F. C., VanBogelen, R. A. and Vaughn, V. (1984) The genetics and regulation of heat-schock proteins. Annu. Rev. Genet. **18**, 295–329
29 Theyssen, H., Schuster, H. P., Packschies, L., Bukau, B. and Reinstein, J. (1996) The second step of ATP binding to DnaK induces peptide release. J. Mol. Biol. **263**, 657–670
30 Buczynski, G., Slepenkov, S. V., Sehorn, M. G. and Witt, S. N. (2001) Characterization of a lidless form of the molecular chaperone DnaK: deletion of the lid increases peptide on- and off-rate constants. J. Biol. Chem. **276**, 27231–27236
31 Mayer, M. P., Schröder, H., Rudiger, S., Paal, K., Laufen, T. and Bukau, B. (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nat. Struct. Biol. **7**, 586–593
32 Pellecchia, M., Montgomery, D. L., Stevens, S. Y., Vander Kooi, C. W., Feng, H. P., Gierasch, L. M. and Zuiderweg, E. R. (2000) Structural insights into substrate binding by the molecular chaperone DnaK. Nat. Struct. Biol. **7**, 298–303

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