Interdomain interaction through helices A and B of DnaK peptide binding domain

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Abstract In order to better define the structural elements involved in allosteric signalling, wild-type DnaK and three deletion mutants of the peptide binding domain have been characterized by biophysical (steady-state and time-resolved fluorescence) and biochemical methods. In the presence of ATP the chemical environment of the single tryptophan residue of DnaK, located in the ATPase domain, becomes less polar, as seen by a blue shift of the emission maximum and a shortening of the fluorescence lifetime, and its accessibility to polar quenchers is drastically reduced. These nucleotide-dependent modifications are also observed for the deletion mutant DnaK1-537, but not for DnaK1-507 or DnaK1-385, and thus rely on the presence of residues 507–537 (helices A and the N-terminal half of B) of the peptide binding domain. These data indicate that αA and half αB contribute to the allosteric communication of DnaK. In the presence of ATP, they promote a conformational change that displaces a residue(s) of the peptide binding domain towards a region of the ATPase domain where the tryptophan residue (W102) is located. A putative role for these helical segments as regulators of the position of the lid is discussed.

Key words: DnaK; Hsp70; Allosteric communication; Fluorescence

1. Introduction

DnaK is a member of the highly conserved 70-kDa heat shock protein family (hsp70) [1,2]. DnaK is the bacterial hsp70 with both constitutive and stress-induced functions. As a molecular chaperone, it binds unstable or unfolded polypeptides, protects them from aggregation and denaturation, and helps them along their folding pathway [2].

DnaK consists of two domains: a 45-kDa N-terminal domain with ATPase activity, and a 25-kDa C-terminal peptide binding domain, whose 3D structure has been solved by X-ray diffraction separately. The highly conserved ATPase domain consists of two large domains divided in two subdomains with a deep nucleotide binding cleft [3]. Two of these subdomains interact with the nucleotide exchange factor GrpE [4], whereas information about the regions of the ATPase domain that interact with the C-terminal domain of the protein is scarce. The peptide binding domain (Fig. 1B) can be divided into a conserved β-sandwich (residues 393–501) and a more variable C-terminal α-helical subdomain (residues 509–607) [5]. The peptide substrate is bound in a cavity formed by the β-sandwich and does not interact with the helical subdomain, that folds over the β-sandwich and is required for the proper locking on of the substrate [6,7]. It has been proposed that the C-terminal helices (residues 538–607) act as a lid that folds around a hydrophobic pocket [5,8]. ATP binding triggers a conformational change transmitted from the ATPase domain to the substrate binding domain, which efficiently releases the bound peptide [9,10]. In the opposite direction, substrate binding to the peptide binding domain stimulates the ATPase activity of the protein [11,12]. However, the molecular mechanism by which the chemical energy of ATP is coupled to the

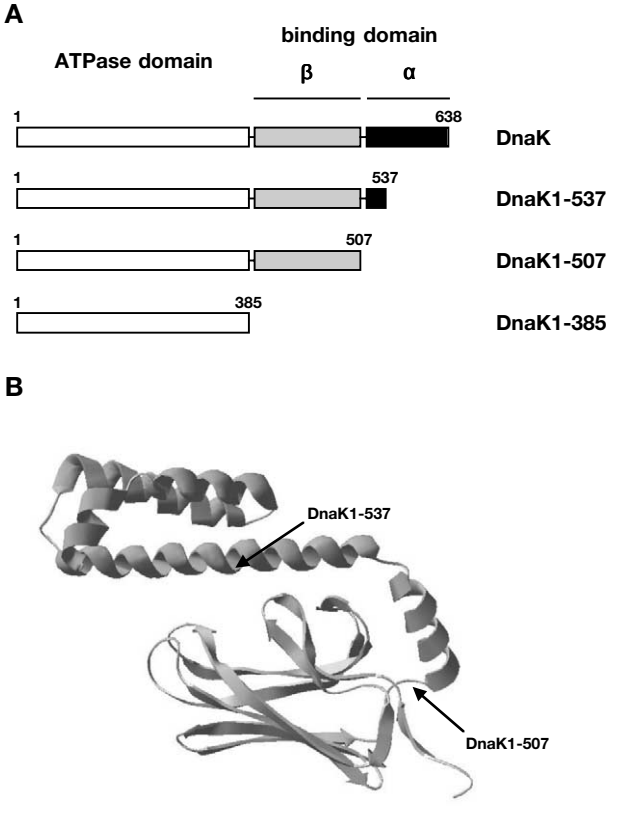

Fig. 1. DnaK deletion mutants. A: Schematic outline of DnaK and the deletion mutants characterized in this study. Relevant residue numbers are indicated. B: Ribbon diagram of the peptide binding domain of DnaK [5] with arrows marking the last residues of mutants DnaK1-537 and DnaK1-507.

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opening and closing of the substrate binding domain and the essential elements for the transmission of the signal are yet poorly understood.

To examine in more detail the allosteric interaction between the ATPase and the substrate binding domains of DnaK, we have characterized by biochemical and biophysical techniques wild-type (wt) and three deletion mutants of DnaK, in which 101, 131 and 253 residues at the C-terminal domain have been deleted. Deletion of these residues progressively eliminates the lid, the connecting α-helices, and the β-sandwich subdomain of the peptide binding domain (Fig. 1A). Our data show that upon ATP binding, the helical elements αA and half αB of this domain are required to displace a residue(s) of the peptide binding domain to a position nearby the single tryptophan (W102) at the ATPase domain. This finding suggests a model in which an allosteric signal is transmitted from the ATPase domain to the connecting helices A and B to promote the displacement of the lid.

2. Materials and methods

2.1. Construction of DnaK deletion mutants

A 2-kb fragment encoding DnaK was amplified by PCR from XL1blue cells using the primers: 5′-CCCGCCATGGGTAAAA-TAATTGGTATCG-3′ (DNAK1N) and 5′-CCCGGATCCAAGCT-TTTATTTTTTGTCTTTGAC-3′. The fragment was separately digested with *Nco*I–*Eco*RI and *Eco*RI–*Bam*HI. The 0.8-kb *Nco*I–*Eco*RI and 1.2-kb *Eco*RI–*Bam*HI fragments were then cloned into *Nco*I–*Bam*HI sites of pTrc99A vector (Amersham Pharmacia). PCR fragments corresponding to the deletion mutants were amplified using the primers: DNAK1N and 5′-CCCGGATCCAAGCTTTTAGTTG-CGAGTCTGTAC-3′ for DnaK1-537; DNAK1N and 5′-CCCGGA-TCCAAGCTTTTACAGACCAGAAGAAGC-3′ for DnaK1-507; DNAK1N and 5′-CCCGGATCCAAGCTTTTAGTCACCAGTCA-GAAC3′ for DnaK1-385. The fragments were cloned in pTrc99A vector as described for DnaK.

2.2. Protein purification

DnaK and the deletion mutants were overexpressed after IPTG induction in BB1553 cells [13], grown at 30°C. Cell extracts were prepared by lysozyme treatment and sonication at 4°C, followed by ultracentrifugation during 30 min. Supernatants were loaded on a Q-Sepharose anion exchange column equilibrated in 20 mM Tris–HCl, pH 7.2, 50 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol (DTT), and eluted with a 4× column gradient from 50 to 500 mM NaCl. Fractions containing DnaK were pooled and, after addition of 5 mM $MgCl_2$, recirculated over a 5-ml ATP-agarose column (Sigma), equilibrated in 20 mM Tris–HCl, pH 7.2, 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$. The column was washed with 2 M NaCl and bound proteins were eluted with 5 mM ATP. This fraction was loaded on a hydroxyapatite column equilibrated in 10 mM Na-phosphate, pH 6.8, 15 mM β-mercaptoethanol and eluted with a 10× column volume from 10 to 500 mM Na-phosphate. Pure DnaK was concentrated and nucleotide was removed by extensive dialysis against 1000 volumes of 20 mM imidazole, pH 7.2, 2 mM EDTA, 10% glycerol. Finally, the protein was dialyzed against 25 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, frozen in liquid nitrogen and stored.

During the purification of ATPase domain 5% glycerol was included in all buffers and an additional gel filtration step was performed. The fraction obtained from the ATP-agarose column was loaded on a Superdex 75 prep grade (Amersham Pharmacia), equilibrated in 25 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 5% glycerol.

2.3. ATPase activity

Steady-state ATPase activity was measured using the assay described by Norby [14]. Assays were performed in 40 mM HEPES, pH 7.6, 50 mM KCl, 11 mM Mg acetate, with 5 μM DnaK, 1 mM ATP at 30°C. Reactions were monitored measuring the absorbance decay at 340 nm for 30 min in a Cary spectrophotometer (Varian).

2.4. Intrinsic fluorescence measurements

Intrinsic fluorescence spectra were recorded on a SLM8100 spectrofluorimeter (Aminco) with excitation at 295 nm and emission at 300–400 nm. Excitation and emission slits were set at 4 nm. The protein and nucleotide concentrations were 5 μM and 0.5 mM, respectively, in 25 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT buffer. In the fluorescence quenching experiments, the emission was measured at 340 nm upon excitation at 295 nm. Acrylamide was gradually added to 3 μM protein in the absence or the presence of 0.5 mM nucleotide. Dilution effects were corrected.

2.5. Time-resolved fluorescence spectroscopy

Radiative decay curves were recorded by the time-correlated single-photon counting technique (Edinburgh Instruments model ηF900). The emission was monitored at 340 nm after excitation at 295 nm by means of a hydrogen flash-lamp with 1.5 ns FWMH pulses and 40-kHz repetition rate. Protein concentration was 5 μM in 25 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT buffer. Nucleotide concentration was 0.5 mM and temperature was 20°C.

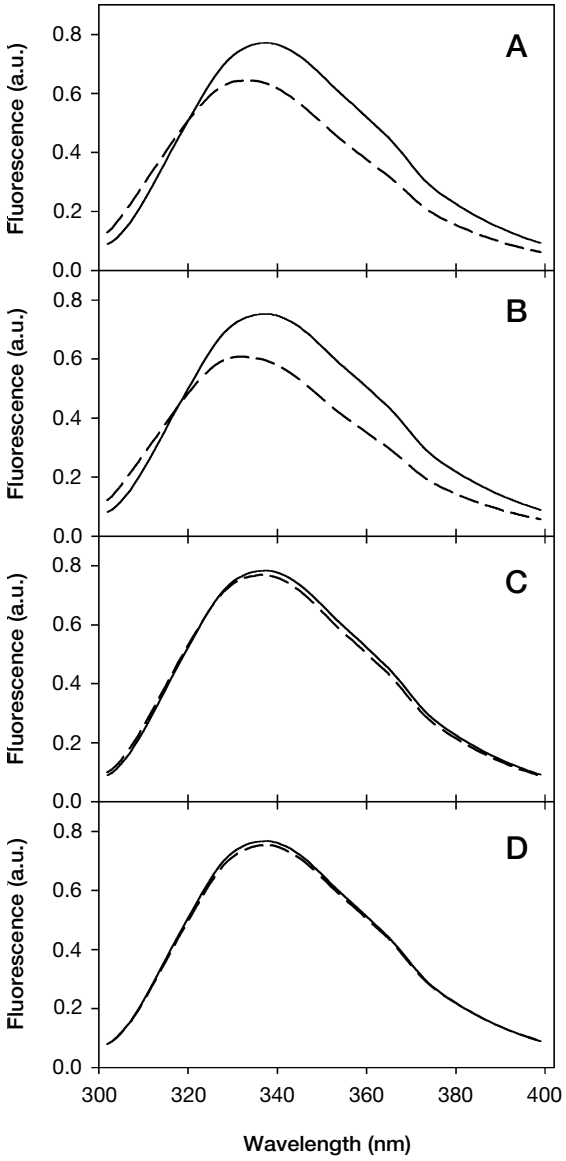

Fig. 2. Fluorescence emission spectra of DnaK and its deletion mutants. Spectra were recorded on samples containing 5 μM protein in the absence of nucleotide (solid line), and presence of 0.5 mM ATP (broken line). A: wt DnaK; B: DnaK1-537; C: DnaK1-507; and D: DnaK1-385. The temperature was 20°C.

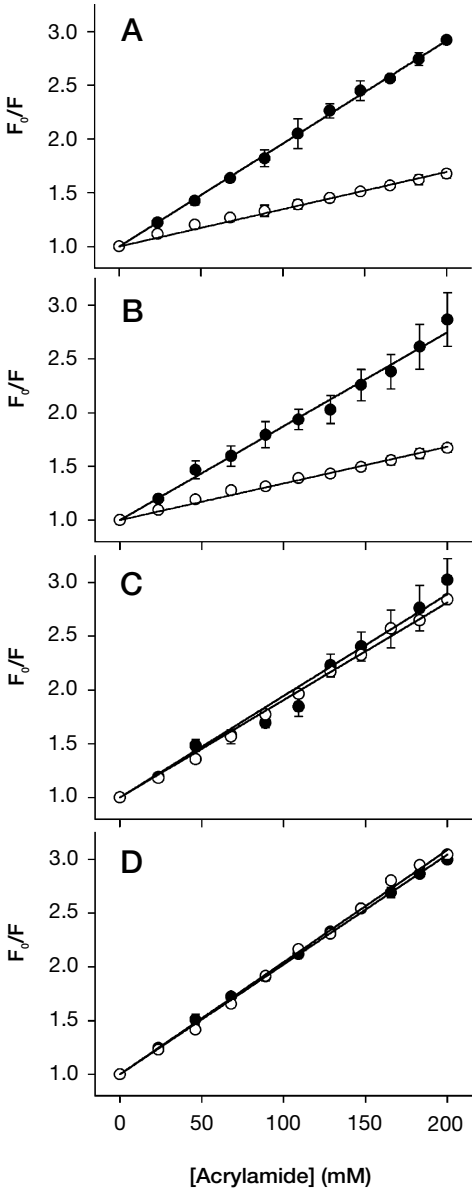

Fig. 3. Stern–Volmer plots of acrylamide quenching of intrinsic fluorescence. A: wt DnaK; B: DnaK1-537; C: DnaK1-507; and D: DnaK1-385. The samples contained 3 μM protein, and were incubated at 20°C in the absence (closed circles) and presence of 0.5 mM ATP (open circles). Data are the average of three independent experiments.

3. Results

3.1. Functional assays

The functionality and allosteric communication of the proteins under study was checked by complementation and peptide-induced ATPase stimulation assays. Wt DnaK and the deletion mutants were grown under IPTG control at non-permissive conditions (40°C) in the BB1553 strain [13]. DnaK1-537 was able to support growth with low efficiency at high IPTG concentrations (>250 μM, data not shown) as reported for a similar DnaK163 mutant protein [6], while wt DnaK completely restored growth at much lower IPTG concentrations (<50 μM).

ATPase activity values obtained for wt DnaK (0.12 mol ATP (mol protein)$^{-1}$ min^{-1}) were in good agreement with previously published data using similar experimental methods [15], and similar to those of the deletion mutants. Binding of peptide NRLLLTG activated by five-fold the ATPase activity of wt DnaK, DnaK1-537 and DnaK1-507, indicating that these mutants maintained allosteric communication (data not shown).

3.2. Steady-state fluorescence spectroscopy

It the presence of ATP the fluorescence of the single Trp residue of DnaK (W102) located in the ATPase domain is quenched, and its emission maximum is shifted from 339 to 334 nm (Fig. 2A) as previously reported [9,15]. These ATP-induced changes required the presence of about 150 residues from the C-terminal domain [9], and thus they can be used as a signature of interdomain interaction. Essentially the same ATP-induced modifications of the fluorescence spectra were observed for DnaK1-537 (Fig. 2B), in agreement with data from the natural mutant DnaK163 (DnaK2-538) [6,9]. In contrast, neither the intensity nor the emission maximum of DnaK1-507 (Fig. 2C) or of the ATPase domain (Fig. 2D) were sensitive to the presence of ATP, the fluorescence spectra of their nucleotide-free, ADP- (not shown) and ATP-bound forms being very similar, with an emission maximum at 339 nm. Therefore, residues 507–537 are required for the above mentioned spectroscopic changes.

3.3. Acrylamide quenching

Acrylamide is a polar, uncharged compound that has the ability to quench any excited tryptophanyl residue that collides with, regardless of whether it is located at the surface or in the apolar core of a protein. Since collisional quenching by acrylamide would depend on the frequency of a Trp residue encountering a quencher molecule, Trp accessibility will be proportional to the degree of quenching. Acrylamide quenching experiments with DnaK and its deletion mutants were performed in the absence and presence of ATP. Stern–Volmer quenching plots (Fig. 3) were linear within the concentration range used, and therefore data were analyzed on the basis of the simple Stern–Volmer equation. The K_{sv} Stern–Volmer constant value found for wt DnaK decreased by three-fold from 9.6 M^{-1} in the absence of nucleotide to 3.2 M^{-1} in the presence of ATP (Fig. 3A; Table 1), in good agreement with previous observations [16]. In the presence of ADP the K_{sv} value was similar to that of free DnaK (not shown). Similar K_{sv} values were obtained for the mutant DnaK1-537 (Fig. 3B; Table 1), suggesting that ATP induced a conformational change in this protein similar to that described for wt DnaK. In contrast, neither DnaK1-507 (Fig. 3C) nor the ATPase domain (DnaK1-385; Fig. 3D) showed the above mentioned ATP-induced decrease in the accessibility of W102 to the

Table 1
Steady-state fluorescence quenching

	Free	+ATP
DnaK	9.64 (±0.11)	3.24 (±0.11)
DnaK1-537	8.79 (±0.29)	3.24 (±0.10)
DnaK1-507	9.90 (±0.45)	9.39 (±0.20)
DnaK1-385	10.23 (±0.12)	10.67 (±0.22)

Apparent Stern–Volmer constants (K_{sv}, M^{-1}) obtained in the absence and presence of 0.5 mM ATP.
K_{sv} were determined from the equation $F_o/F = 1 + K_{sv} \times$[acrylamide]. Data are the average of at least three independent experiments on two different protein batches.

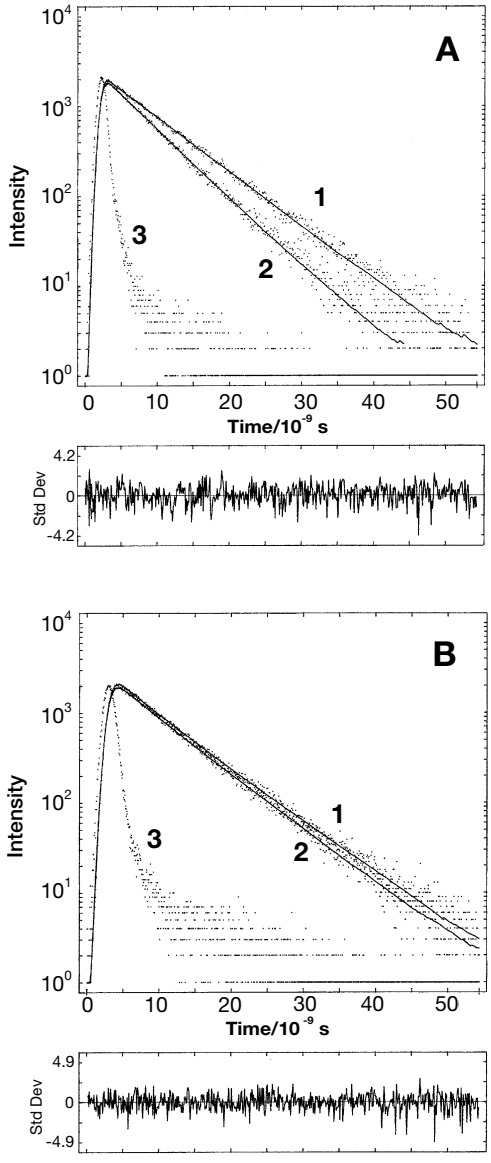

Fig. 4. Fluorescence decay of DnaK (A) and DnaK1-507 (B). The points represent experimental data and the solid line the best decay–time fit. The standard deviation of the fit is shown below each panel. In both panels, trace 1 is obtained in the absence of nucleotide, trace 2 in the presence of 0.5 mM ATP, and trace 3 corresponds to the lamp pulse profile. Excitation was set at 295 nm, and emission was monitored at 340 nm. Measurements were performed at 20°C.

quencher (Table 1), their K_{sv} values being identical regardless of the presence of nucleotide. It should be mentioned here that the nucleotide concentration is around 1600-fold higher than the protein concentration, and that nucleotide hydrolysis does not significantly modify the ATP concentration during the time-course of the experiment (15 min). Taken together, these data indicate that residues 507–537 mediate allosteric communication between domains that results in a decreased accessibility of W102 to polar quenchers in the presence of ATP.

3.4. Time-resolved fluorescence spectroscopy

The time-dependent excited-state decay of tryptophan fluorescence is very sensitive to the environment, which makes it a useful probe of protein structure and dynamics [17]. The lifetime decay of wt DnaK could be accurately fitted with two components of 6.95 and 0.46 ns, respectively, the contribution of the long-lifetime component being by far greater (96.2%) than that of the short-lifetime component (3.8%) (Fig. 4A; Table 2). Given the low contribution of this component to the intensity decay ($<6\%$) in all proteins studied, the experimental data will be interpreted in the light of the long-lifetime component. Addition of ADP did not significantly modify either the lifetime of the components or their fractional amplitudes (Table 2). However, in the presence of ATP the fluorescence decay was faster and the long-lifetime component was significantly shortened (5.45 ns) (Fig. 4A; Table 2). Essentially the same results were obtained with DnaK1-537 (Table 2). As expected from the previous data, the ATP-induced shortening of the long-lifetime component was not detected in DnaK1-507 (Fig. 4B; Table 2) or DnaK1-385 (Table 2), being the observed long lifetimes and fractional intensities the same, within experimental error, in the presence and absence of nucleotides.

4. Discussion

Allosterism is a key mechanism by which chaperones regulate their function. The fine tuning of interdomain interactions couples nucleotide binding with co-chaperone interaction and substrate release, and allows productive folding cycles.

It has been demonstrated that removal of the complete α-helical domain at the C-terminus of DnaK results in a protein that retains the ATP-induced peptide release and the peptide stimulation of the ATPase activity ([7,18] and this work). These data suggest that allosteric communication between the ATPase domain and the adjacent β-subdomain of the peptide binding domain occurs, and that this communication modulates substrate affinity and ATPase activity. However, the lidless mutants have altered peptide binding kinetics [6,7]

Table 2
Time-resolved fluorescence

	Free					ATP					ADP				
	τ_1 (ns)	α_1	τ_2 (ns)	α_2	χ^2	τ_1 (ns)	α_1	τ_2 (ns)	α_2	χ^2	τ_1 (ns)	α_1	τ_2 (ns)	α_2	χ^2
DnaK	6.95	0.962	0.46	0.038	1.06	5.45	0.967	0.59	0.033	1.01	6.78	0.961	0.55	0.039	1.22
DnaK1-537	6.86	0.971	0.58	0.029	1.03	5.47	0.962	0.42	0.038	1.18	7.01	0.948	2.00	0.052	1.04
DnaK1-507	6.99	0.940	2.24	0.060	1.13	6.70	0.961	2.00	0.039	1.19	6.79	0.971	1.43	0.029	1.02
DnaK1-385	6.86	1	–	–	1.16	6.94	1	–	–	1.09	7.03	1	–	–	1.06

Fluorescence intensity decay curves were recorded and fitted to a double (DnaK, DnaK1-537 and DnaK1-507), or single exponential function (DnaK1-385).
Lifetime (τ), contribution (α) and the fitting parameter (χ) are given.

and are not able to complement the deletion of wt DnaK ([7,18] and this work). It is clear that the lid (residues 538–607) is required to efficiently lock on the substrate in the peptide binding pocket. Thus, peptide release will require a displacement of the lid to open the binding cavity, the molecular mechanism by which the lid is removed from the peptide binding pocket being at present unknown. The fluorescence spectroscopy data shown here monitor an interdomain interaction mediated by a specific segment of the helical subdomain of the peptide binding domain that could be relevant for the displacement of the lid. It was previously shown that ATP induces a blue shift of the fluorescence emission maximum and requires the presence of 150 residues of the C-terminal domain [9]. Our data localize more accurately the sequence, within the peptide binding domain of DnaK, responsible for the interaction with the ATPase domain that induces the above mentioned spectroscopic changes. This sequence comprises residues 507–537, forming helix A and the first half of helix B [5] of wt DnaK (Fig. 2). In a recent study it has been shown that the fluorescence of DnaK2-517 is sensitive to the presence of peptide substrate and ATP [19], which might help to better define the residues responsible for the nucleotide-induced fluorescence changes.

As judged by the 3D structure of the ATPase domain [3,4], the indole ring of the single tryptophan (W102) is predicted to be highly accessible to the solvent, in agreement with the high K_{sv} value obtained for the DnaK1-385 mutant, a protein devoid of interdomain interactions. The apparent K_{sv} values estimated for the free and ADP-bound states of wt DnaK and the mutants DnaK1-537, DnaK1-507 are similar to those of the ATPase domain (DnaK1-385), indicating that in these conformations W102 is also highly accessible to polar quenchers. However, ATP promotes a three-fold decrease in the K_{sv} values of wt DnaK and DnaK1-537, but not in DnaK1-507 (Fig. 3). Therefore, αA and the N-terminal half of αB are responsible for the reduction of the accessibility of W102 in the ATP-bound state of wt DnaK. The same dependence on the presence of these helical segments is found for the shortening of the fluorescence lifetime upon ATP binding, most likely due to a residue(s) moving closer and quenching W102. The interpretation of these results is not straightforward, since they might be caused either by a direct contact between αA–αB and the region of the ATPase hosting W102, or by a conformational change mediated by these helical segments that could displace other regions of the peptide binding domain towards the ATPase domain. In any case, the data presented here demonstrate that αA and the N-terminal half of αB contribute to the allosteric communication between DnaK domains.

It is interesting to note that the X-ray structure of the substrate binding domain indicates that helix A and the first half of B form a folding unit with the β-subdomain, since their buried side chains do interact, while the other helical elements, forming the lid, fold around a separate hydrophobic core [5]. αA and the N-terminal half of αB might function as the transmission belt to displace the position of the lid relative to the β-subdomain, most likely through the hinge in the middle of αB, and mediate opening of the peptide binding cavity in the presence of ATP. Helix B spans both subdomains and thus its interaction with the ATPase domain might be critical to modulate the position of the lid. This mechanism would be advantageous since it provides a way to transmit the information from the ATPase domain to both subdomains of the substrate binding domain, which in turn would result in a destabilization of the peptide–DnaK complex and a concomitant displacement of the lid, promoting an efficient peptide release.

The biological relevance of αA and αB has also been described in the mitochondrial Hsp70 (mtHsp70), a close homologue of DnaK. mtHsp70 interacts with Tim44, a component of the mitochondrial inner membrane translocase, in an ATP-dependent manner to promote preprotein translocation across the mitochondrial membranes. Similarly to what is found for DnaK, deletion of αA and αB impaired the nucleotide-dependent dissociation from Tim44 [20,21]. Therefore, helices A and B are involved in the regulation of the interaction of mtHsp70 with Tim44. The role of αA and αB as allosteric elements connecting both protein domains might be a common structural element of Hsp70s, regardless of their functional diversification.

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