Ionic Contacts at DnaK Substrate Binding Domain Involved in the Allosteric Regulation of Lid Dynamics*

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To gain further insight into the interactions involved in the allosteric transition of DnaK we have characterized wild-type (wt) protein and three mutants in which ionic interactions at the interface between the two subdomains of the substrate binding domain, and within the lid subdomain have been disrupted. Our data show that ionic contacts, most likely forming an electrically charged network, between the N-terminal region of helix B and an inner loop of the ionic contacts, most likely forming an electrically charged network, within the lid subdomain have been disrupted. Our data show that ionic interactions between the two subdomains of the substrate binding domain, and among residues in the lid subdomain that are part of the hinge region involved in the ATP-induced allosteric displacement of the lid away from the peptide binding site. These interactions also stabilize peptide–Hsp70 complexes at physiological (37 °C) and stress (42 °C) temperatures, a requirement for productive substrate (re)folding.

The ubiquitous Hsp70 chaperone proteins are essential to prevent aggregation of misfolded proteins under heat shock conditions. They also participate in cellular processes such as folding of newly synthesized polypeptides, protein translocation across membranes, assembly and disassembly of protein complexes, and refolding of protein aggregates (1).

Hsp70 proteins consist of two major structural domains: a conserved N-terminal ATPase domain and a more variable C-terminal substrate binding domain (SBD).4 The SBD contains a β-sandwich, which holds the peptide binding groove, and an α-helical subdomain that folds over the β-structure and might control accessibility of the peptide binding site. High resolution three-dimensional structures of isolated forms of both structural domains with different bound ligands have been reported (2–11). Recently a model of a two-domain truncated Hsp70 from Thermus thermophilus has been proposed based on NMR studies (12). Some of the interdomain contacts are better revealed in the x-ray structure of a bovine Hsc70 that lacks most of the helical subdomain at the SBD (13). In DnaK, the best known bacterial representative of the Hsp70 family, the substrate peptide is bound in an extended conformation between two loops that protrude from the β-sandwich subdomain (L1,2 and L3,4), and are in turn buttressed by two other loops (L4,5 and L3,6). The helical subdomain of the SBD acts like a lid over the β-sandwich subdomain. The α-helices A and B are packed onto the β-sandwich, and helix B covers the substrate binding cavity. The distal part of helix B, together with helices C, D, and E, builds up a hydrophobic helical core that acts as a “latch” (6, 9). Although interdomain coupling occurs in the absence of the lid and the bound peptide contacts the β-structure but not the α-helical subdomain, its presence has been confirmed to be essential in stabilizing Hsp70-substrate complexes (14, 15), especially at stress temperatures (16).

The broad spectrum of activities that Hsp70 proteins exhibit requires their interaction with hydrophobic segments of (partially) unfolded polypeptide chains, in a nucleotide-controlled unknown mechanism (17). When ATP binds the ATPase domain of Hsp70s, an allosteric signal is transmitted to the SBD that adopts a conformation with fast substrate association and dissociation rates and, therefore, low substrate affinity. In contrast, the substrate is tightly bound to the ADP-bound conformation, which displays a high affinity for substrates (18).

One of the most important characteristics of DnaK conformations, that has been proposed to dictate their affinity for peptides, is the position of the lid relative to the β-subdomain of the SBD. The displacement of the lid away from the peptide binding site seems to facilitate substrate dissociation (18). Therefore, analysis of the interactions that anchor the lid to the β-subdomain at the SBD might be relevant to localize the hinge regions of the protein that allow lid movement. Among these interactions, those in which helix B is involved seem to be especially relevant, because residues at this helical segment interact with amino acids belonging to both SBD folding units (6). Among these interactions, those in which helix B is involved seem to be especially relevant, because residues at this helical segment interact with amino acids belonging to both SBD folding units (6). Among these interactions, those in which helix B is involved seem to be especially relevant, because residues at this helical segment interact with amino acids belonging to both SBD folding units (6).

EXPERIMENTAL PROCEDURES
Site-directed Mutagenesis of DnaK and Purification of Proteins

Mutants DnaK1A, DnaK2A, and DnaK3A were obtained by sequential PCR steps using DnaK flanking primers (19) and mutagenic primers (underlined letters show the nucleotides changed for each mutation): A,
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Partial Proteolysis by Trypsin

Partial proteolysis of wild-type DnaK and mutants was carried out in 40 mM Hepes buffer, 50 mM KCl, 10 mM MgCl₂ (pH 7.6). 5 μM Hsp70 proteins were incubated at 30 °C with 1.6 units of trypsin, in the absence or presence of 1 mM ATP or ADP. Aliquots containing 5 μg of protein were taken at different incubation times and diluted directly into Laemmli loading buffer for 12.5% SDS-PAGE analysis.

Mass Spectrometry Analysis

In-gel Digestion—Selected tryptic bands were cut from Coomassie Blue-stained, fixed gels and subjected to in-gel tryptic digestion according to Shevchenko et al. (26) with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH₄HCO₃ and 12.5 ng μl⁻¹ trypsin (Roche Diagnostics, recombiant, proteomic grade trypsin, Penzberg, Germany) in an ice bath. After 30 min the supernatant was removed and discarded, and 20 μl of 50 mM NH₄HCO₃ was added to the gel piece, the digestion proceeding at 37 °C overnight. Supernatant was recovered, and peptides were extracted twice: first with 25 mM NH₄HCO₃ and acetonitrile (ACN) (Romil, Cambridge, UK) and second with 0.1% trifluoroacetic acid and ACN. Supernatants of each sample were pooled and dried in a SpeedVac. Prior to MS analysis, pellets were resuspended in 10 μl of 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany) for MALDI-MS and in 10 μl of 0.1% formic acid (Merck) for ESI-MS analysis and sonicated for 5 min.

Sample Preparation for MALDI-MS Analysis—Recovered peptides were purified prior to MALDI analysis using customized nano-columns as described by Gobom et al. (27) with some modifications. A column consisting of 100–300 nl of activated charcoal from Sigma (C-5510) was packed in a constricted GEloader tip (Eppendorf, Hamburg, Germany) (28). A 1-ml syringe was used to force liquid through the column by applying air pressure. The column was equilibrated and washed with 0.1% trifluoroacetic acid, and bound peptides were subsequently eluted directly onto the MALDI target with 0.5 μl of a solution of α-cyano-4-hydroxycinnamic acid (20 μg μl⁻¹ in ACN:trifluoroacetic acid, 70:30, v/v).

MALDI-MS Analysis—Peptide mass fingerprinting was performed on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany). Positively charged ions were analyzed in reflector mode, using delayed extraction. The spectra were obtained by randomly scanning the sample surface. Typically 200–300 spectra were averaged to improve the signal-to-noise ratio. Spectra were externally calibrated resulting in a mass accuracy of <70 ppm. Protein identification was performed by searching in a non-redundant protein data base (NCBI) using the Mascot searching engine (www.matrixscience.com). The following parameters were used for data base searches: one missed cleavage was allowed, and carbamidomethylation of cysteine and oxidation of methionine were selected as fixed and variable modification, respectively.

ESI-MS Analysis—Tandem electrospray mass spectra were acquired using a MicrO-Q-TOF (Waters Corp., Milford, MA) mass spectrometer interfaced to a CapLC capillary chromatograph. 7 μl of sample was loaded onto a Symmetry300 C18 NanoEase Trap precolumn (Waters) and washed with 0.1% formic acid for 5 min at a flow rate of 20 μl min⁻¹. Precolumn was connected to an Atlantis dC18 NanoEase column (Waters) equilibrated in 5% ACN, 0.1% formic acid. A flow splitter was used to decrease the flow rate to 200 nlnmin⁻¹, and peptides were eluted with a 30-min linear gradient from 10% to 60% ACN directly to the nano-electrospray capillary tip (New Objective, Woburn, MA). The capillary voltage was set to 1750 V, and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4

5′-GVVGAAGCTGCCGTAAGTTG-3′ and B, 5′-CTTACGGGCA-GCTTGGCGGG-3′, for DnaK1A (D526A); C, 5′-CGGCCATCTGGCT-CAACACCCGTCAGTTGAAG-3′ and D, 5′-GGCA-CGCTGCTGAGCAGATGGGCCCTGG-3′, for DnaK2A (D540A/K548A); E, 5′-GAAGCCTGTTGACTCAGCTGCAACCAGGGCG-3′; F, 5′-GTTGCGCAGTCTGTAACCCAGGGCTTCAAAC-3′; G, 5′-CACAGACCCGCTAAGCAGGTTAGAAG-3′ and H, 5′-CTGCTTTACGCCGTGCTGTCAG-3′ for DnaK3A (E531A/R536A/ R547A). PCR products were cloned into the bacterial expression vector pTrc99A (Amersham Biosciences) as described previously (19) and sequenced to verify the encoded proteins. DnaK wild-type and mutants were expressed, purified, and extensively dialyzed to obtain nucleotide-free samples (20).

DnaJ and GrpE were expressed in BL21 cells and purified as described elsewhere (21, 22). Protein concentration was determined by the colorimetric Bradford assay (Bio-Rad).

Peptide Binding

F-APPY peptide (fluorescein-CALLQSRLLSAPRRAAATARY) binding experiments were performed in 25 mM Hepes, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol. To ensure equilibrium, mixtures containing 35 mM F-APPY and different Hsp70 concentrations (from 1 nM to 50 μM) were incubated at overnight at 4 °C. Fluorescence anisotropy data were recorded on a SLM8100 spectrofluorometer (Aminco) with excitation at 492 nm, emission at 516 nm, and slit widths of 8 nm. The fraction of peptide bound for each Hsp70 protein concentration was calculated, and the data were fitted as described previously (23, 24). Peptide binding kinetics were analyzed at 25 °C, 37 °C, and 42 °C using F-APPY and Hsp70 concentrations of 35 nM and 1 μM (wt DnaK, DnaK1A, and DnaK2A) or 3 μM (DnaK3A) Hsp70, respectively, as well as 0.5 mM ADP to stabilize the ATPase domain at stress temperatures. Data were fitted to a monoexponential equation consistent with a bimolecular reaction and the kobs value was plotted against Hsp70 concentration to obtain the binding parameters k⁺ and k⁻ as described (14, 24).

Circular Dichroism

Far-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a thermoelectric cell holder. The collection parameters were 0.5-mm path length, 1 nm bandwidth, 50 nm min⁻¹ scan rate, and 0.5-s response time. Scans were performed at 20 °C in 10 mM sodium borate (pH 9.0) and 0.3 mg ml⁻¹ protein concentration. Spectra were averaged from 10 scans and normalized for protein concentration. For the thermal stability experiments, the mean residue ellipticity at 222 nm was monitored using a 2-mm path length cell, and temperature was increased from 20 °C to 90 °C at a scanning rate of 60 °C h⁻¹.

Differential Scanning Calorimetry

DSC was performed in a VP-DSC microcalorimeter (MicroCal, Northampton, MA). Protein samples were extensively dialyzed against 25 mM glycine, pH 9.0, centrifuged to remove aggregates, and degassed prior to be loaded in the calorimeter. At pH 9.0 the solubility of the thermally denatured conformation is high enough to make the denaturation transition highly reversible (25). Experiments were carried out under positive pressure to avoid degassing during heating. The calorimetric data were analyzed using ORIGIN software provided with the calorimeter. Protein concentration was 1–2 mg ml⁻¹ and the scanning rate was 60 °C h⁻¹. Experiments were carried out at least twice on two different batches of each protein.

Partial proteolysis of wild-type DnaK and mutants was carried out in 40 mM Hepes buffer, 50 mM KCl, 10 mM MgCl₂ (pH 7.6). 5 μM Hsp70 proteins were incubated at 30 °C with 1.6 units of trypsin, in the absence or presence of 1 mM ATP or ADP. Aliquots containing 5 μg of protein were taken at different incubation times and diluted directly into Laemmli loading buffer for 12.5% SDS-PAGE analysis.
over a survey m/z range of 400–1500. Obtained spectra were searched against the Swiss-Prot data base using ProteinLynx Global Server 2.1 (Waters). For protein identification the following parameters were adopted: complete carbamidomethylation of cysteines; 25 ppm of peptide mass tolerance; 0.05-Da fragment mass tolerance; and 1 missed cleavage. When the presence of a particular peptide on a sample was checked, an inclusion list of m/z of the precursor was used for the MS/MS acquisition.

**Functional Assays**

**ATPase Activity**—Steady state ATPase activity was assayed in 40 mM Hepes, pH 7.5, 50 mM KCl, 11 mM magnesium acetate buffer at 30 °C, as described previously (19). Protein and ATP concentration were 3 μM Hsp70 and 1 mM, respectively. Reactions were followed measuring the absorbance decay at 340 nm for 30 min in a Cary spectrophotometer (Varian). In the peptide and co-chaperone stimulation assays, NRLLLTG (NR) peptide was added at a final concentration of 0.3 mM, and DnaJ and GrpE were added at 0.3 and 0.9 μM respectively, as previously described (29).

**Refolding of Denatured Luciferase**—Luciferase was denatured for 45 min at 25 °C, in 6 μl guanidine HCl, 100 mM Tris, pH 7.7, 10 mM dithiothreitol. Refolding was achieved by diluting luciferase to 25 nM in 50 mM Tris, pH 7.7, 55 mM KCl, 15 mM MgCl₂, 5.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, containing an ATP-regenerating system (4 mM phosphoenolpyruvate and 20 ng/ml pyruvate kinase) and 6 μM Hsp70, 6 μM DnaJ, and 7.2 μM GrpE. Reactivation was initiated by addition of 5 mM ATP. Luciferase activity was measured after a 90-min reactivation period at at 25 °C, 37 °C, or 42 °C, using the luciferase assay system (Promega E1500) in a Sinergy HT (Biotek) luminometer.

**RESULTS**

**Design of Mutants**—Analysis of the ionic interactions at the SBD reveals that in six of the ionic contacts found, one of the involved residues is located in helix B. This helical segment communicates the helical and β-subdomains participating, via its N-terminal region, in the folding unit formed by the β-sandwich and helix A, and through its C-terminal region, in the second folding unit formed by the remaining helical segments of the lid subdomain (6). To analyze the effect of these ionic interactions on the ATPase activity of DnaK, three mutants were designed: DnaK1A, DnaK2A, and DnaK3A. These mutants were generated by site-directed mutagenesis, and their secondary and tertiary structures were characterized by NMR spectroscopy.
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contacts in the structural and functional properties of DnaK, each corresponding residue in helix B was substituted by alanine (Fig. 1A). It is important to note that none of the mutated residues were located in the B-sandwich subdomain, because this might modify the peptide substrate-binding properties of the protein. In the first mutant, DnaK(D526A), named DnaK1A, a salt bridge between the N-terminal half of helix B and the inner loops of the B-sandwich subdomain is disrupted. The second mutant, DnaK2A, bears two mutations (D540A/K548A) that break a salt bridge and a hydrogen bond between the C-terminal part of helix B and the outer loops of the B-subdomain. These interactions are part of the so-called "latch," that is believed to control the accessibility of the peptide binding site (1, 6, 13, 16, 30, 31). The third mutant, DnaK3A, holds three substitutions (E531A/R536A/R547A) that hamper formation of two salt bridges and a hydrogen bond between residues at helix B and helices C and D of the lid. The main difference between these proteins is that mutations in DnaK1A and DnaK2A modify the interaction between helix B and the B-sandwich subdomain, whereas those in DnaK3A might alter the interaction between helix B and the lid subdomain.

Multiple alignment of Hsp70 sequences reveals the conservation degree of residues mutated in this study (Fig. 1A). It is important to note that none of the mutated residues were located in the B-sandwich subdomain, because this might modify the peptide substrate-binding properties of the protein. In the first mutant, DnaK(D526A), named DnaK1A, a salt bridge between the N-terminal half of helix B and the inner loops of the B-sandwich subdomain is disrupted. The second mutant, DnaK2A, bears two mutations (D540A/K548A) that break a salt bridge and a hydrogen bond between the C-terminal part of helix B and the outer loops of the B-subdomain. These interactions are part of the so-called "latch," that is believed to control the accessibility of the peptide binding site (1, 6, 13, 16, 30, 31). The third mutant, DnaK3A, holds three substitutions (E531A/R536A/R547A) that hamper formation of two salt bridges and a hydrogen bond between residues at helix B and helices C and D of the lid. The main difference between these proteins is that mutations in DnaK1A and DnaK2A modify the interaction between helix B and the B-sandwich subdomain, whereas those in DnaK3A might alter the interaction between helix B and the lid subdomain.

Multiple alignment of Hsp70 sequences reveals the conservation degree of residues mutated in this study (Fig. 1A). Although Asp$^{526}$/Arg$^{445}$ is widely conserved among the Hsp70 family members, the ionic contacts at the latch are not equally conserved, and those within the lid show the lowest conservation degree.

Interaction with Peptide Substrates—The ability of wt and the three mutants to bind peptide substrates was analyzed by measuring the change in anisotropy of a fluorescence labeled peptide (F-APPY). In the presence of wt DnaK, the anisotropy value increases as the peptide binds DnaK, as previously reported (16, 23, 32). Equilibrium binding curves were obtained with increasing protein concentrations, and the anisotropy increase for each protein was fitted to a single site binding model (Fig. 2 and Table 1). The mutants, particularly DnaK1A and DnaK3A, show a slightly decreased affinity for F-APPY (Table 1).

The kinetic parameters $k_{\text{on}}$ and $k_{\text{off}}$ were also estimated by following F-APPY binding at increasing protein concentrations. The data were fitted to a single exponential compatible with a single site binding model (Table 3). From the linear plots of $k_{\text{on}}$ versus protein concentration, $k_{\text{on}}$ ($y$-intercept) and $k_{\text{off}}$ (slope) were obtained (Fig. 3B). These parameters are clearly different for the mutants analyzed here, as seen in Table 1. Although the kinetic constants of wt and DnaK3A are very similar, those of DnaK2A are 1.4- to 2-times higher, and the single mutation in DnaK1A induces a dramatic increase of the association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$). The $K_d$ values estimated from these kinetic parameters for wt DnaK, DnaK2A, and DnaK3A also indicate that their affinity for F-APPY is comparable. In the case of DnaK1A, the binding process is too fast to obtain a reliable $K_d$ value from kinetic measurements with our experimental time resolution (see Fig. 3A). However, a rough estimation of the kinetic constants can be achieved. This behavior is similar to that observed for a DnaK lidless mutant (14, 16).

As expected for a bimolecular reaction, increasing temperatures enhance the rate of DnaK-peptide complex formation, the final anisotropy value being slightly lower at higher temperatures (Fig. 4, A and B). When the same experiment is performed with DnaK2A and DnaK3A, essentially the same temperature dependence of the peptide anisotropy is observed. It should be noted that the final protein concentration was adjusted (1 μM for wt DnaK, DnaK1A, and DnaK2A; 3 μM for DnaK3A, Fig. 2A) to account for the differences in affinity, so that >90% of the
added peptide substrate was bound. The stability of the substrate-protein complex against the thermal challenge is clearly different for DnaK1A mutant. In this case, the amount of bound peptide diminishes with temperature in such a way that at stress temperatures this mutant is unable to stably bind F-APPY (Fig. 4, A and B). Cycling the sample between 25 °C and 42 °C induces reversible changes in the anisotropy of the DnaK1A-peptide mixture (Fig. 4C), indicating that the temperature-induced conformational transition responsible for the change in affinity of DnaK1A for F-APPY is reversible, as described for a lidless mutant of DnaK (16).

Secondary Structure and Stability of wt DnaK and the Mutants—The secondary structure of wt DnaK and the three mutants studied here was analyzed by CD spectroscopy. As shown in Fig. 5A, a comparison of the shapes and ellipticity values suggests that mutant proteins adopt a secondary structure similar to that of wt DnaK and, therefore, that mutations do not alter this structural level. To analyze whether the thermal stability of the protein was modified by the mutations, the ellipticity at 222 nm was measured as a function of temperature. Similar melting curves were obtained for wt DnaK, DnaK1A, and DnaK2A mutants (Fig. 5B). They do display transitions with similar amplitudes at ~43 °C and 70 °C, as previously reported for the wild-type protein (33). In contrast, the melting curve of DnaK3A displays a transition at ~45 °C, whereas that at ~70 °C is hardly detected. It should be noted that the ellipticity value at 90 °C is similar for all proteins indicating that their thermally denatured conformation is also similar.

Protein stability was also characterized by DSC, which provides the thermodynamic parameters of the unfolding process. The thermogram of wt DnaK (Fig. 6) shows three transitions that have been previously assigned (25). The lowest temperature transition comes from the unfolding of the ATPase domain, the second peak is due to the denaturation of the substrate binding domain, and the high temperature one contains contributions from the denaturation of both protein domains. The DSC profiles of all mutants differ considerably from that of wt
The thermogram of DnaK1A reflects a destabilization of the ATPase and substrate binding domains evidenced by a decrease of the temperature and calorimetric enthalpies of the other peaks are, as compared with DnaK1A, slightly affected (Fig. 6 and Table 2). To further prove that the low temperature endotherm in the DSC profile of DnaK1A was due to the denaturation of the ATPase domain, the same experiment was carried out in the presence of 1 mM ADP. At this ligand concentration, only the \( T_m \) of the nucleotide binding domain of wt DnaK is upshifted by 9 °C (25), as it is observed for DnaK1A (data not shown), thus indicating that the above assignment also applies to this single point mutant.

Disruption of the ionic interactions at the latch in DnaK2A also modifies the thermal denaturation profile of the protein, although to a lesser extent. The only significant difference is a 27% decrease in the enthalpy of the second transition that is also downshifted (\( \Delta T = 2.5 ^\circ C \)), whereas the temperature and calorimetric enthalpies of the other peaks are, as compared with DnaK1A, slightly affected (Fig. 6 and Table 2). The thermal profile of DnaK3A shows that the temperatures of the first and second transitions are slightly shifted in opposite directions, their calorimetric enthalpy values being similar to those of the corresponding transitions of wt DnaK (Fig. 6 and Table 2). However, the enthalpy of the third transition is reduced by 65% suggesting that, as found for a DnaK deletion mutant lacking helices C-E (16), denaturation of these helices that fold around a common hydrophobic core (6) gives rise to an endotherm at \(~75 ^\circ C\) (Fig. 6 and Table 2). The low enthalpy value is also in agreement with the weak intensity of the high temperature transition detected by CD for DnaK3A.

| Table 2 | Thermodynamic parameters associated to the thermal denaturation of DnaK and the alanine exchanged mutants |
|---------|--------------------------------------------------------------------------------------------------|
|         | \( T_m \) | \( \Delta H_{cal} \) | \( T_m \) | \( \Delta H_{cal} \) | \( T_m \) | \( \Delta H_{cal} \) |
| wt DnaK | 43.5 | 79 | 57.5 | 94 | 74 | 72 |
| DnaK1A  | 45.7 | 28.1 | 51 | 61 | 73.6 | 75 |
| DnaK2A  | 43.9 | 66 | 55 | 68 | 75.6 | 57 |
| DnaK3A  | 46.2 | 90 | 54 | 98 | 76.5 | 24 |

In contrast to wt DnaK, trypsin treatment processes almost all the ADP state of DnaK1A at the shortest incubation time analyzed (1.5 min), generating major fragments of \(~56~and 46~kDa\) that at longer times are converted into a 44-kDa one. Interestingly, this difference regarding the fragment pattern of the ADP states of wt DnaK and DnaK1A is not observed for their ATP states, the tryptic pattern being very similar for both proteins. Minor differences are seen for the 36- and 33-kDa fragments that regardless of the bound nucleotide are not detected with DnaK1A, instead two protein species of 18 and 16 kDa are observed.

A fragment similar to that of 56 kDa was attributed to tryptic cleavage around residue 500 of DnaK (20), therefore the appearance of the 56-kDa fragment at short incubation times of DnaK1A suggests that the C-terminal \( \alpha \)-helices are more accessible to enzymatic cleavage in this mutant. This hypothesis has been confirmed by MS analysis of the 56-kDa major fragment, showing that it arises most likely from cleavage...
at amino acids Lys$^{514}$ or Arg$^{517}$ in helix A of the lid subdomain, because in contrast to wt DnaK, which shows a good sequence coverage (83%) in the C-terminal helical subdomain, peptides beyond Lys$^{514}$ are not found in the 56-kDa fragment (Fig. 7, B and C). The good agreement between the apparent molecular mass estimated from SDS-PAGE (56 kDa) and amino acid sequence (55.477 kDa and 55.896 kDa) for the 1–514 or 1–517 fragments, respectively, further supports this interpretation. The absence of fragments of 36 and 33 kDa in the pattern of nucleotide-bound DnaK$^1$A is likely due to further tryptic cleavage within its C-terminal region, as seen by MS analysis and in agreement with previous data (20). This sort of cleavage will also generate the low molecular weight fragments observed in DnaK$^1$A.

The fragment patterns of both nucleotide-bound conformational states of DnaK$^2$A are the same to those of wt DnaK. However DnaK$^3$A displays different tryptic sites. In the presence of ADP, DnaK$^3$A generates a major fragment of ~62 kDa that at intermediate incubation times is also seen together with a fragment of ~57 kDa and others already described for wt DnaK (see above, Fig. 7A). MS/MS analysis of the fragments detected only in DnaK$^3$A (i.e. those with apparent molecular masses of 62 and 57 kDa, labeled in Fig. 7B as "b" and "c," respectively) are compatible with protein molecules comprising residues 1–577 or 1–581 and 1–527, which will have molecular masses of 62.204 and 62.733 and 56.906 kDa, respectively, in good agreement with the apparent values estimated from SDS-PAGE (Fig. 7, B and C). ATP induces a conformational change in DnaK$^3$A evidenced by the disappearance of the 62-kDa fragment at short times and, as seen for wt DnaK, the appearance of the 53-kDa species. This might indicate that the ATP-induced conformational changes would protect the tryptic site that generates the 62-kDa fragment at the C-terminal helix bundle. Note that residue Lys$^{581}$ is the ionic partner of residue Glu$^{531}$ replaced by alanine in DnaK$^3$A, and that residue Lys$^{577}$ is located in its close vicinity.

Binding of ATP to DnaK quenches the fluorescence of its single Trp residue and shifts its emission maximum toward lower wavelengths (19, 20). These spectroscopic changes require the interaction of both protein domains and therefore reflect allosteric communication. As already described for wt DnaK, ATP binding to the three mutants induces a similar 4 nm blue-shift of their emission maxima and a 15% reduction of the fluorescence intensity (data not shown). These data suggest that the environment of Trp$^{102}$ undergoes similar modifications in all proteins during the ATP-induced conformational changes.

**Functionality of DnaK Mutants**—To further assess the functional consequences of the structural differences presented so far, the effect of different ligands on the ATPase and refolding activities of wt DnaK and the three mutants was analyzed. The timing of the ATPase cycle has been proposed as an important factor that modulates the biological activity of DnaK and other Hsp70s (15), because it controls the transition between the substrate-accepting and -releasing states of the protein and is therefore regulated by co-chaperones and peptide substrates (17, 29, 35). The effect of NR peptide, GrpE, and DnaJ on the ATPase activity of wild-type and DnaK mutants is illustrated in Fig. 8A. Each of these
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**DISCUSSION**

ATP binding to the DnaK ATPase domain generates an allosteric signal that is transmitted to both subdomains of the SBD (18). The allosteric conformational change most likely lowers the affinity of the β-subdomain for peptide substrates and modifies the position of the lid to allow peptide dissociation. The concerted allosteric structural rearrangement of both subdomains could be stabilized, particularly at stress temperatures, by a set of interactions between both subdomains and/or between any of these subdomains and the ATPase domain. Therefore, identification of the interactions involved in maintaining the location and/or dynamics of different protein regions is necessary to understand the functional cycle of DnaK. This is the goal of this work, where the contribution of ionic contacts at the interface of the two subdomains of the SBD, i.e. between residues at helix B and amino acids at the lid or β-sandwich, to the conformational and functional properties of DnaK is analyzed.

Disruption of the highly conserved ionic pair Asp^{526}–Arg^{445} has a strong effect on the conformation, stability, and functional properties of the protein. Essential to describe this effect are the interactions that the N terminus of αB establishes with the inner loop L<sub>4,5</sub>. A detailed inspection of this protein region reveals that Asp^{526} interacts not only with Arg^{445} but also with Lys^{446}, forming a “charged network” (Fig. 9). These types of charged networks have been found to stabilize protein structures (37). In the case of DnaK it might be important to fix the position of the C-terminal α-helices relative to the β-sandwich subdomain in the ADP state of the protein. This assumption is corroborated by experimental evidence showing that disruption of these interactions controls important structural and functional properties of DnaK. One piece of evidence is related to the enhanced trypsin accessibility of the interface between the C-terminal subdomains in the ADP-bound state of DnaK1A. A similar observation has been made for the apo-state of a Hsc70 mutant in which the counterion of Asp^{526}, Arg^{447}, was replaced by Gly (38). Our results also indicate that the higher susceptibility to proteolytic degradation might be due to differences in lid structure and/or dynamics in the ADP state of DnaK. If this were the case, the accessibility of the peptide binding site should be modified, as experimentally observed for both the association and dissociation constants of a peptide substrate, as previously described for DnaK(DS26N) (39). This interpretation is further supported by a similar decrease in the thermal stability of the peptide-protein complexes observed for DnaK1A and a lidless mutant lacking helices B–E (16). This similarity also points to the possibility that other interactions between helix B and the β-sandwich might not be properly established if the ionic contacts in this protein region are disrupted.

Although DnaK1A possesses a significantly destabilized peptide binding site, faster substrate association, and dissociation rates (Figs. 3

**FIGURE 8.** Functionality of DnaK and mutants. A, stimulation of the ATPase activity by peptide and co-chaperones. Steady-state ATPase activity was measured at 30 °C at a protein and ATP concentrations of 3 μM and 1 mM, respectively. NR (NRLLTGT) peptide, DnaJ, and GrpE were added at 300 μM, 0.3 μM, and 0.9 μM, respectively. B, luciferase refolding activity. Reactivation of GndHCl-denatured luciferase by wt DnaK and mutants DnaK1A, DnaK2A, and DnaK3A at 25, 37, and 42 °C was achieved as described under “Experimental Procedures.” Control refers to refolding in the absence of chaperones.

**FIGURE 9.** Network of ionic interactions in which D526A is involved (PDB: 1DKX). Asp^{526} at the N-terminal region of helix B establishes two ionic contacts with residues Arg^{445} and Lys^{446} at the inner loop L<sub>4,5</sub>. This triad may also stabilize other contacts between the β-sandwich and helices A and B, as indicated. The atomic distances, measured by WHAT IF software (40), between selected charged amino acid residues are given in Angstroms.
and 4), and higher stimulation of its ATPase activity by peptides and co-chaperones when added together (Fig. 8A), its folding activity was only compromised at 37 °C and not at 25 °C (Fig. 8B). It can therefore be assumed that the stability of the peptide-Hsp70 complex could control the chaperone function of the mutant and that co-chaperones do not stabilize the complex at physiological (37 °C) temperature. Under these experimental conditions, the substrate released from an unstable complex would join the irreversible aggregation process that kinetically competes with productive folding. It is interesting to note that the 3-fold higher co-chaperone-mediated ATPase stimulation of DnaK1A also occurred at 37 °C (data not shown) and that only at 25 °C the protein was able to refold denatured luciferase. It follows from this comparison that a normal refolding activity of this protein can coexist with an accelerated ATPase cycle, suggesting that both activities are not directly coupled.

The second effect of this mutation (D526A) is related to the reduction of the thermal stability of the β-sandwich subdomain and the ATPase domain of the protein, most likely due to modifications of interdomain interactions in which both subdomains of the SBD are involved (Figs. 5 and 6). Supporting this interpretation is the fact that a single substitution in DnaK1A destabilizes to a larger extent both domains of the apo-protein, as compared with the other two mutants. This finding might be a consequence of mutating a protein region that holds stabilizing interdomain interactions. In fact, both the NMR-derived model (12) and the x-ray structure (13) of two-domain truncated Hsp70 analogues propose the region formed by helix A and the N terminus of helix B as an interdomain contact site in the absence of ligands. Indeed, αA and the N terminus of αB are required to observe the ATP-induced fluorescence changes of Trp102 located at the subdomain IB of the DnaK ATPase domain (19). In summary, disruption of the ionic interaction in DnaK1A alters the conformation and/or dynamics of the lid in the ADP state of the protein and modifies interdomain interactions responsible for the stability of both protein domains. The involvement of this structural region in both interdomain interactions and contacts between the subdomains of the SBD might couple ATP binding to the NBD with lid displacement and peptide dissociation. Moreover, the interaction of J co-chaperones with this contact site (13, 39) could be part of the structural mechanism by which J proteins regulate the functionality of both Hsp70 domains.

It is interesting to note that the conformational change brought about by this single amino acid replacement specially affects the ADP-bound state of the protein, i.e. the high affinity conformation for substrates, whereas the ATP state is similar to that of wt DnaK as judged by partial proteolysis and fluorescence spectroscopy. This might reflect that this protein region could be part of the functional hinge that allows anchoring of both SBD subdomains in the ADP state, and the displacement of the lid associated to the ATP state of the protein. The functional cycle of DnaK has been analyzed assuming that in the ADP and ATP states the substrate binding site closes and opens, the rate of opening and closing being the main difference between both states (18). Two mechanisms have been proposed to explain the movement of the lid away from the peptide binding site that characterizes the open conformation. The first one is based on the two different lid conformations found in the x-ray structure of DnaK SBD (6). In one of them, the long helix B that folds over the binding site was kinked in the middle, suggesting that a hinge at this position of helix B allows the upwards movement of the lid. The second mechanism is supported by the different positions of the α-subdomain relative to the β-subdomain seen in two NMR analysis of truncated forms of Hsc70 and DnaK SBDs (4, 5) and a crystal structure of truncated HscA SBD (11). Despite the limitations of the NMR structures that lack helices C–E and show a largely unfolded helix B that interacts with the peptide binding site, these structures suggest that the helical subdomain can occupy different positions with respect to the β-subdomain and that opening of the peptide binding site could also be achieved by moving the entire helical subdomain. Our data support the view that the highly conserved interactions (see Fig. 1B) between the N terminus of helix B and loop L4 of the β-sandwich subdomain form part of the pivotal region that would function as a hinge to move the entire lid away from the binding site. Moreover, these interactions could be essential in defining the position of the lid in one of the two physiologically relevant conformations, the ADP-bound, high affinity state for substrates, and therefore to define the lid movements during the functional cycle of the protein at 37 °C. If this were the case, movement of the entire α-helical subdomain might require modulation of other interactions, besides those mentioned above. Among these interactions are the ionic contacts between helix B and both subdomains of the SBD that are lost in DnaK2A and DnaK3A. Regarding mutations affecting to the latch in DnaK2A, it is interesting to note that they do not have as much impact in protein conformation (Fig. 7A), peptide association kinetics (Fig. 3 and Table 1), thermal stability of the peptide-DnaK2A complex (Fig. 4), and in the stability of the protein structure (Figs. 5 and 6) as does the single mutation introduced in DnaK1A. However, the interactions forming the latch are critical in maintaining a functional DnaK cycle, because their disruption renders the protein unable to refold protein substrates under the experimental conditions used in this study. At present we cannot explain this behavior.

The three ionic interactions disrupted in DnaK3A have the expected effect on the compactness of the C-terminal α-helical region, namely a lower thermal stability (Fig. 6 and Table 2) and an enhanced accessibility to tryptic cleavage (Fig. 7). Interestingly, this accessibility is reduced in the presence of ATP indicating a conformational change in the C-terminal helix-bundle upon ATP binding, at least in mutant DnaK3A, that to our knowledge had not been previously detected in wild-type DnaK. The modifications introduced in DnaK3A affect slightly the peptide binding properties of the protein (Figs. 2–4), but they do not alter the functionality of the mutant (Fig. 7), indicating that the compactness of the C-terminal helix-bundle is not essential for the DnaK refolding activity.

In summary, the results presented here demonstrate that the ionic contacts between the N-terminal region of helix B and the β-sandwich subdomain are necessary to stabilize the interaction between the lid and the β-subdomain in the ADP-bound state, which in turn controls the stability of the protein–substrate complex especially at high temperatures and, therefore, the functional properties of DnaK. We propose that these interactions could be part of the functional hinge that regulates lid movements in Hsp70 proteins in response to ATP binding.

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