The Lid Subdomain of DnaK Is Required for the Stabilization of the Substrate-binding Site*

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We examined the effect of deletion of different segments in the helical subdomain (the so-called “lid”) of the DnaK peptide-binding domain on peptide binding and protein stability. At 25 °C, wt DnaK and the deletion mutant proteins are able to stably bind peptides with similar affinity. However, at physiological (37 °C) and stress (42 °C) temperatures, removal of the N-terminal half of αβ and the rest of the lid drastically decreases the ability of the protein to bind substrates. Differential scanning calorimetry and infrared spectroscopy show that this behavior is accompanied by destabilization of the peptide-binding domain. Our data suggest that the reversible interaction between the lid and β-sandwich subdomains of DnaK peptide-binding domain is required for the stabilization of the loops that form the peptide-binding site, which in turn modulates the protein affinity for peptide substrates. This interaction might have functional implications because it could prevent rebinding of the peptide substrate, which would be forced to fold.

DnaK is the bacterial Hsp70 chaperone with both constitutive and stress-induced functions. Among different biological activities, DnaK is engaged in preventing the aggregation of thermally denatured proteins, thus becoming essential for cell viability under heat-shock conditions (1). It consists of two domains, a 45-kDa N-terminal ATPase domain and a 25-kDa C-terminal substrate-binding domain (2). The ATPase domain is a bilobed structure that contains the nucleotide-binding site in a channel between the two lobes (3). The substrate-binding domain is composed of two subdomains, each with characteristic structural and most likely functional properties. The β-sandwich subdomain holds the hydrophobic substrate-binding cavity, and it is followed by an α-helical subdomain that consists of five antiparallel helices (4). This subdomain acts as a lid, folding over the β-sandwich subdomain without contacting the peptide substrate (4).

It has been postulated that during the functional cycle of DnaK, the protein adopts two conformational states, one with high affinity for the peptide substrate, which maintains the substrate locked in the binding cavity, and another one consisting of an open conformation in which the lid is displaced, leaving the binding cavity uncapped (4). Peptide dissociation would require the destabilization of the DnaK substrate complex and thus the weakening of the hydrophobic interactions that stabilize it in a polar environment.

The role of the lid in the functional cycle of DnaK is far from being understood. It has been shown that it is not involved in interdomain coupling (5), although the lid is essential in maintaining long-lived substrate-DnaK complexes, because in its absence, ATP-induced peptide dissociation is significantly accelerated (6, 7). In this context, a recent work indicates that helix D is engaged in maintaining stable DnaK-peptide complexes, and that helix E and the last 31 residues at the C terminus might contact the ATPase domain (8). Furthermore, the finding that helix A and the C-terminal half of helix B are involved in interdomain communication (9) points to the importance of the lid subdomain in maintaining the functionality of DnaK. Besides these functions, it has also been proposed that the lid of DnaK and other Hsp70 proteins might interact with DnaJ (10).

The interaction between the two subdomains of the substrate peptide-binding domain is stabilized through a network of hydrogen bonds and several salt bridges (4). The structural and functional consequences of these interactions are unknown. It has been reported that the overall fold of the β-sandwich is not significantly modified in the absence of the lid, although the above mentioned interactions seem to stabilize the conformation of the loops that build the substrate-binding site (5, 11). Following this suggestion, the role of the lid subdomain in maintaining the ability of the protein to stably bind substrates at physiologically relevant temperatures has been analyzed using several deletion mutants (Fig. 1). Our data indicate that the interactions between the lid and the β-subdomain are responsible for the ability of the protein to bind peptide substrates at physiological (37 °C) and stress (42 °C) temperatures. A mechanism is proposed in which the displacement of the lid coupled to the destabilization of the substrate-binding site upon ATP binding might weaken the hydrophobic interactions that stabilize the complex, so that the peptide would be efficiently released to a polar medium and substrate rebinding would be prevented.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—DnaK and the deletion mutants were expressed in the BB1553 strain and purified as described (9).

ATPase Activity—Steady-state ATPase activity was measured as in Ref. 9. Assays were performed in 40 mM Hepes, pH 7.6, 50 mM KCl, 11 mM magnesium acetate, 1 mM ATP, and 5 μM DnaK. Reactions were monitored at 30 °C measuring the absorbance decay at 340 nm for 30 min in a Cary spectrophotometer (Varian). In the peptide stimulation...
assays, NRLLLTF (NR) peptide was added at 500 μM concentration.

Peptide Binding—Peptide binding was carried out in 25 mM HEPES, pH 7.6, 50 mM KCl, 0.5 mM ADP, 5 mM MgCl₂, 1 mM dithiothreitol. Fluorescein-CALLQSR LLASAPRRAATARY (peptide F-APPY) and DnaK concentrations were 35 nM and 1 μM, respectively, to ensure 100% binding at 25 °C. Binding was started by the addition of F-APPY to the protein solution thermostated at the desired temperature. Fluorescence anisotropy was continuously measured on an SLMS100 spectrofluorometer (Aminco) with excitation at 492 nm, emission at 516 nm, and 8 nm slit widths.

Differential Scanning Calorimetry (DSC)—Differential scanning calorimetry was performed in a VP-DSC microcalorimeter (Microcal, Northampton, MA). Prior to DSC experiments, samples were dialyzed against 25 mM sodium borate, pH 9.0, and concentrated on Microcon-30 (Amicon) filters to 30–40 mg/ml. The filtrates obtained in the last concentration step were used as references. Samples were placed in a thermostated cell, between two calcium fluoride windows separated by 50 μm (D₂O) or 6 μm (H₂O) spacers. Infrared spectra were recorded in a Nicolet Magna spectrometer equipped with an MCT detector. Data acquisition and analysis were performed as described previously (12). Temperature was controlled by a thermostate in direct contact with the windows.

RESULTS

Functionality of the Different DnaK Variants: ATPase Activity—To investigate the functionality of the mutants, the steady-state and peptide-stimulated ATPase activities were determined. The ATPase activity of the DnaK1–554 mutant (0.42 mol ATP (mol protein)⁻¹ min⁻¹) was higher than those of wt DnaK and the other deletion mutants, which were found to be similar to the published value of 0.12 mol ATP (mol protein)⁻¹ min⁻¹ (9). This result might be caused by a cis-interaction of hydrophobic residues at the C-terminal half of helix B with the peptide-binding site, as was shown recently for a similar construct (8). DnaK1–554 was stimulated only 2-fold by NR, because of its high basal activity, in contrast to the 5-fold activation observed for wt DnaK, DnaK1–537, and DnaK1–507. Despite this difference, the activity of the peptide-induced activated state was similar for all of the proteins (0.62 mol ATP (mol protein)⁻¹ min⁻¹).

Peptide Substrate Binding: Effect of Temperature—The binding kinetics of a fluorescently labeled peptide to wt DnaK and to the above-mentioned deletion mutants were followed at different temperatures. To choose the appropriate experimental conditions, the affinity of each protein variant for peptide F-APPY was estimated as described previously (13). At 25 °C, the estimated dissociation constants (Kd) were 0.11, 0.24, and 0.20 μM, for wt DnaK, DnaK1–537, and DnaK1–507, respectively. Therefore, protein and peptide concentrations were fixed so that more than 95% of the peptide was bound at 25 °C. As shown in Fig. 2A (upper panel), F-APPY binding to wt DnaK was faster at higher temperatures.

Unfortunately, DnaK1–554 could not be included in this type of experiments, because the high peptide concentration required to displace part of the lid from the peptide-binding site hampers data analysis. Both DnaK1–537 (data not shown) and DnaK1–507 (Fig. 2A, bottom panel) bound F-APPY faster than DnaK at 25 °C, as reported previously for lidless mutants (6). However, their ability to bind the peptide was significantly reduced at physiological temperatures (37 °C) and almost abolished at heat-shock temperatures (42 °C) (Fig. 2, A and B). The conformational change responsible for the temperature-induced decrease in the F-APPY affinity of these mutants was reversible, because heating the samples from 25 to 42 °C promoted peptide dissociation, and cooling them back to 25 °C completely restored peptide binding (Fig. 2C). Taken together, these data indicate that the lid is an important structural element to stabilize the DnaK-peptide complex against the thermal challenge.

Protein Stability and Conformation: DSC Studies—The wt DnaK thermogram showed three endotherms centered at 43.5, 57.5, and 74 °C (Fig. 3A; Table I). Although the first and second peaks have been associated with the unfolding of the 45-kDa N-terminal, ATPase domain and of the 25-kDa substrate-binding domain, respectively, the high temperature transition contains contributions from both protein domains (14). The thermodynamic parameters derived from the DSC profiles of wt DnaK agree very well with previously reported data (Table I; Ref. 14). Deletion of 85 residues at the C terminus of the protein, corresponding to helices C–E and the last 31 unre-
solved residues (DnaK1–554), slightly modified the temperature and enthalpy of the low temperature transitions. However, it decreased substantially the enthalpy of the high temperature peak (Fig. 3B; Table I). The latter effect was also observed in the DnaK1–537 mutant, in which the C-terminal half of residues 539–554 was deleted (Fig. 3C). The thermogram corresponding to this mutant showed in addition a decrease in the temperature of the second transition (ΔT ≈ 4.7 °C), with a 30% reduction in the denaturation enthalpy (Table I). The denaturation enthalpy of this transition was further reduced in the DnaK1–507 mutant (Fig. 3D; Table I). The thermal unfolding profile of the ATPase domain showed two endotherms centered at 47.5 and 75.8 °C (Fig. 3E; Table I), as reported previously (14).

**IR Spectroscopy: Assignment of the Amide I Band Components**—IR spectroscopy has been shown to be a useful tool to characterize both the conformational properties and stability of proteins (15, 16). We first analyzed the IR spectra of wt DnaK and its deletion mutants with the aim of assigning the major spectral features. The original and deconvolved IR spectra of wt DnaK in H2O and D2O buffers at 20 °C are shown in Fig. 4A. The original spectra displayed the absorption maximum at 1650 and 1640 cm⁻¹ in H₂O and D₂O buffer, respectively. These findings, and the fact that the intensity of the amide II band at 1550 cm⁻¹, due mainly to N-H bending vibrations, was drastically reduced upon deuteration, are in good agreement with previous studies (17). Overlapping bands within the amide I band (1700–1600 cm⁻¹) can be resolved by deconvolution of the original spectra. The deconvolved spectra showed several components that can be assigned to turns (H₂O, 1673 and 1668 cm⁻¹; D₂O, 1678 and 1668 cm⁻¹), α-helix (1650 cm⁻¹), and extended structures (1688 and 1629 cm⁻¹). The most interesting deuteration-sensitive spectral feature was the change in the relative intensities of the components at 1650 and 1640 cm⁻¹. This behavior could be due to aperiodic structures such as loops (18, 19) and/or solvent-exposed helical conformations (20). Considering the x-ray structure of the ATPase and substrate-binding domains, both possibilities might actually occur.
and progressive deletion of the α and β subdomains of the peptide-binding domain should help to distinguish between these alternatives. Thus the IR spectra of DnaK1–507 and of the ATPase domain (DnaK 1–385) were characterized in H2O and D2O buffers (Fig. 4B). As compared with wt DnaK, the DnaK1–507 spectra recorded in either solvent were similar regarding the number and position of the component bands. However, the intensity ratio of the band components at 1650/1640 decreased in H2O (from 0.7 to 0.4) and increased in D2O (from 0.8 to 1.4) buffer upon lid deletion. This result indicates that the helical structure forming the C terminus of the DnaK peptide-binding domain contributes to the absorbance at 1650 and 1640 cm−1 in H2O and D2O, respectively, a behavior characteristic of solvent-exposed helical conformations. Following a similar comparison strategy between DnaK and the ATPase domain, it is clear that the β-sandwich subdomain that holds the substrate-binding pocket is the main structure responsible for the band components at 1688 and 1629 cm−1, because its deletion eliminates these vibrations from the spectrum (Fig. 4B).

Spectroscopic Characterization of the Thermal Denaturation of wt DnaK and Deletion Mutants—To describe in more detail the conformational changes associated with the thermal events observed by DSC, the temperature-dependence of the IR spectra of wt DnaK and the deletion mutants was analyzed. The IR spectrum corresponding to the thermally denatured protein recorded at 82 °C showed a broad band at 1646 cm−1 and a shoulder at 1676 cm−1 (Fig. 5A). Although the former indicated the presence of unordered structures, the latter arose from turns remaining in the denatured structure. Similar band components have been described for thermally denatured RNase (21) and plastocyanin (18). We did not observe aggregation of the thermally unfolded protein as in a previous work (17), most likely because of the different experimental conditions (pH 9.0) and to the significantly lower protein concentration used in this study. Positive and negative features in the temperature-induced IR difference spectra reflected fine structural differences between conformations obtained at consecutive temperatures (every 1–2 °C) during the heating procedure (Fig. 5B). In the 20–52 °C temperature range, when (according to DSC data) the ATPase domain unfolds, differential signals at 1676 (+)/1653 (−) and 1640(−)/1629(+) were observed above 38 °C, indicating a temperature-induced change in the protein secondary structure. The former is probably caused by the unfolding of the peripheral helical conformation of the ATPase domain, which could convert at least partially into turns, and the latter may be related to solvent exchange in the core β-strands of this domain. This interpretation is supported by the presence of a negative band at 1550 cm−1, arising from unexchanged NH groups of the protein, in the same temperature interval. The difference spectra obtained in the 52–64 °C temperature range, at which peptide-binding domain denatures according to DSC, showed a broad differential signal at 1673(+)/1640(−) (Fig. 5B). The upward position of the positive component of this differential signal indicated that this conformational change is not due to solvent exchange, as reflected by the absence of a negative signal at 1550 cm−1. Both solvent-exposed helical or loop conformations could contribute to the observed absorbance change at 1640 cm−1. However, a predominant contribution from helical elements can be excluded, considering that the thermal unfolding of wt DnaK did not show a pronounced change in the ellipticity value at 222 nm in the same temperature range (22). Finally, in the 64–80 °C interval (Fig. 5B), the difference spectra showed a feature at 1665(+)/1629(−), representing mainly the unfolding of the remaining β-structure.

To compare in an easier way the results obtained with wt DnaK and the different deletion mutants, the intensities of four differential signals (1650, 1640, 1629, and 1550 cm−1) were represented as a function of temperature (Fig. 6). These profiles revealed three temperature intervals at which variations of the above-mentioned intensities occurred. These intervals correspond rather closely to the three endotherms detected by DSC. The four differential signals underwent changes in the temperature interval corresponding to the denaturation of the ATPase domain (38–52 °C). At intermediate and high temperatures, only the 1640 and 1629 cm−1 differential signals were significantly affected (Fig. 6A). The 1640 cm−1 signal was of particular interest because, as shown above, it arises from both solvent-exposed α-helices at the lid subdomain and loops from the peptide-binding domain. Denaturation of the lid subdomain took place in the 64–80 °C temperature range, because its deletion induced the disappearance of the absorbance changes at 1640 cm−1 (Fig. 6, A–C). We should note that a resolved differential signal at 1640 cm−1 is not clearly observed in Fig. 5B for wt DnaK because of overlapping with the more intense differential band at 1629 cm−1. The remaining contributions from loop structures to the 1640 cm−1 signal were observed in the 52–64 °C temperature range (Fig. 6A), as was the endotherm corresponding to the denaturation of the peptide-binding domain. Although deletion of αC–E and the last 31 residues did not significantly modify this signal (Fig. 6, A and B), removal of αA-B downshifted this spectral feature, which merged with those originating from the unfolding of the ATPase domain (Fig. 6C), in good agreement with DSC data. Similar results were observed for DnaK1–537 (not shown). These findings reinforced the idea that the interaction between both subdomains of the peptide-binding domain stabilized loop conformations against the thermal challenge. When the same data were analyzed for the ATPase domain, it was evident that the high temperature transition monitored by the 1629 cm−1 signal disappeared (Fig. 6D) and, therefore, it was assigned to denaturation of the β-sandwich.

DISCUSSION

Besides other biological functions, DnaK binds and prevents aggregation of polypeptide substrates under stress conditions and during protein folding (1, 2). The binding site of DnaK...
consists of a hydrophobic cavity, which accommodates a single
hydrophobic side chain, formed by two pairs of inner and outer
loops that protrude from the \( \beta \)-sandwich, and an arch formed
by residues Met-404 and Ala-429 (4). Although the helical lid
does not contact the bound substrate, it might control the
accessibility of the hydrophobic-binding cavity (4, 6–8). The
DnaK-substrate complex is stabilized through \( i \) hydrogen
bonds between the backbone of two pocket-forming loops of
DnaK and the peptide backbone that recognizes the extended
conformation of the peptide, \( ii \) hydrophobic contacts with
approximately five consecutive residues of the substrate, and
\( iii \) Van der Waals interactions between side chains of residues
lining the binding cavity and of the peptide substrate (4). Muta-
tional studies have revealed that the most important con-
tribution to substrate binding comes from the hydrophobic
interactions (23). As inferred from the available structures of
the binding domain complexed with peptide substrate (4, 5, 11),

**Fig. 4.** IR spectroscopy. A, IR spectra of DnaK at 25 °C in aqueous
and deuterated buffer (25 mM sodium borate, pH 9.0 and pD 8.6,
respectively). \( \text{Tin lines, original spectra; thick lines, deconvolved spec-
tra. Deconvolution was performed using a Lorentzian bandwidth of 18
\text{cm}^{-1} \) and a resolution enhancement factor of 2. Protein concentra-
tion was 40 mg/ml (\( \text{H}_{2}\text{O} \)) and 20 mg/ml (\( \text{D}_{2}\text{O} \)). B, comparison of the amide I
region of the deconvolved IR spectra of DnaK, DnaK1–507, and DnaK
ATPase domain. Spectra were recorded at 25 °C in aqueous and deu-
terated buffer and deconvolved as in A.

**Fig. 5.** Temperature-induced difference IR spectra of DnaK. A,
IR spectra of DnaK recorded in deuterated buffer (25 mM sodium borate,
pD 8.6) at 20 (upper traces) and 80 °C (lower traces). \( \text{Tin lines, original
spectra; thick lines, deconvolved spectra. Deconvolution was performed
as in Fig. 4. Protein concentration was 20 mg/ml. B, difference IR
spectra of DnaK. The sample in deuterated buffer was heated from 20
to 82 °C, at 60 °C/h, and spectra were recorded every 1–2 °C during the
process. Infrared difference spectra were obtained by subtracting two
original spectra recorded at consecutive temperatures.
binding and release of substrates would depend on (i) the helical lid that folds over the binding cavity, (ii) the so-called arch, which encloses the peptide backbone, and (iii) the hydrophobic central pocket. Therefore, peptide binding and release would rely on interactions that stabilize the complex and on the displacement of the lid, relative to the \( \beta \)-sandwich.

In this context, an important finding of this work is that the lid is required for DnaK to stably bind peptide substrate under physiological conditions (e.g. 37 °C). Our data clearly demonstrate that, in the absence of the C-terminal half of \( \alpha B \), \( \alpha C \), \( \alpha D \), \( \alpha E \), and the last 31 residues at the C terminus, the ability of DnaK to bind peptide substrates is drastically reduced at physiological temperatures (37 °C) and virtually abolished at heat-shock temperatures (42 °C). To discuss these data, we shall first consider the interactions between each of the deleted blocks in the mutants and the rest of the protein. According to the x-ray structure of the peptide-binding domain, helices \( \alpha C-E \) form together with the C-terminal half of \( \alpha B \) an autonomous hydrophobic core that can be considered as a folding unit, whereas the buried side chains of the N-terminal half of \( \alpha B \) and \( \alpha A \) do interact with the \( \beta \)-sandwich. As far as the stability is concerned, deletion of \( \alpha C-E \) mainly affects the events taking place at high temperatures, which have been associated with conformational changes involving solvent-exposed helical conformations and \( \beta \)-structure. Deletion of these helical segments causes the disappearance of the high temperature transition monitored by the 1640 cm\(^{-1}\) band, which might be related to the reduction of the enthalpy of the high temperature endotherm observed by DSC. Unfortunately, we were unable to characterize the ability of DnaK1–554 to bind substrates, most likely because of the interaction of hydrophobic residues at the C-terminal helix B with the peptide-binding site, as shown for similar constructs (8, 24).

The C-terminal half of \( \alpha B \), the segment that is additionally deleted to generate DnaK1–537, interacts with the outer loops of the \( \beta \)-subdomain forming what is known as the latch. A salt bridge between Asp-540 and Arg-467 and hydrogen bonds between several residues (His-544, Lys-549, Asp-431) stabilize the latch. As experimentally observed and well established for many proteins (25, 26), disruption of a salt bridge would have a destabilizing effect on protein conformation, as would a weaker hydrogen bonding, although to a lesser extent. Comparison of the x-ray structure of the substrate-binding domain and the NMR structure of the isolated \( \beta \)-sandwich (4, 5) does indeed reveal that the mobility of the outer loops of the \( \beta \)-sandwich is significantly higher in the latter, suggesting that the interactions mentioned above are involved in the stabilization of the outer loops. Experimentally, both IR and DSC show that the peptide-binding domain is destabilized against the thermal challenge upon deletion of the C-terminal half of \( \alpha B \) (DnaK1–537 mutant). Moreover, the 1640 cm\(^{-1}\) IR signal, which is greatly shifted to lower temperatures in this mutant, contains mainly contributions from loop conformations. As found with the isolated \( \beta \)-sandwich subdomain (5), mutants lacking these interactions are able to stably interact with the peptide F-APPY at 25 °C, in good agreement with the similar overall structure found for this subdomain isolated in solution, or as part of the whole substrate-binding domain (4). However, our data show that at physiological (37 °C) and stress (42 °C) temperatures, the absence of the lid might increase the flexibility of the outer loops that form the binding site and reduce the affinity of DnaK for peptide substrates. The same argument might explain the 10-fold decrease in the affinity for peptide substrates of the isolated \( \beta \)-sandwich, as compared with wt DnaK (5). The fact that all protein species studied bind F-APPY at 25 °C and release it upon ATP binding (not shown) indicates that interdomain communication affects both subdomains of the peptide-binding domain. Only at 37 and 42 °C does the interaction between these subdomains significantly modify the stability of the substrate-binding site.

Although extrapolation of the results presented here to the functional mechanism of wt DnaK is not straightforward, our data might help the understanding of how peptide substrates are bound and released by DnaK. The flexibility of the substrate-binding site detected by NMR suggests that DnaK binds substrates following an “induced fit” pattern. In the closed state of DnaK, the latch would stabilize the conformation of the outer loops and, therefore, of the binding site, so that the substrate would stably interact with the protein. In response to the allosteric action of ATP, a conformational change of the \( \beta \)-sandwich subdomain and a displacement of the position of the lid relative to the \( \beta \)-sandwich, as proposed previously (4), would occur. A displacement of the lid would very likely break the interactions at the latch that, as our results suggest, would destabilize the outer loops, thus weakening the hydrophobic

![Fig. 6. Temperature dependence of selected infrared differential signals. Intensities of differential signals at 1550 (Δ), 1629 (□), 1640 (◇), and 1650 (○) cm\(^{-1}\) for DnaK (A), DnaK1–554 (B), DnaK1–507 (C), and DnaK ATPase domain (D), as a function of temperature.](http://www.jbc.org/Downloaded from)
DnaK-substrate complex. These conformational events would promote the dissociation and release of the peptide substrate from the binding cavity. This hypothesis might also help to understand how DnaK prevents rebinding of the released substrate which is still in a non-native conformation and which would halt protein folding to the native state. Several mechanisms have been put forward to answer this important question: (i) DnaJ dissociates from the DnaK-substrate complex before nucleotide exchange (27, 28); (ii) in the presence of ATP, two different protein conformations exist, one of which would release the substrate, whereas a conformational change of DnaK leading to the second conformation would enable DnaK to rebind substrates (29); and (iii) DnaK modifies the conformation of the substrate so that it cannot rebind to the chaperone (30). Our data suggest that the different conformations that the outer loops forming the binding cavity might adopt in the closed and open states could control the affinity of DnaK for peptide substrates. In the ATP-bound, open conformation, the unstable loops would lower the affinity of DnaK for peptide substrates, thus preventing rebinding and forcing the polypeptide chain to fold.

A highly dynamic mechanism for the steady-state operation of DnaK-binding domain has been proposed (23), in which the substrate-binding cavity opens and closes periodically in both the ADP and the ATP states: the rates of opening and closing that control the accessibility to the binding site might be associated with a reversible conformational change of the outer loops, which, in turn, modulates the affinity of the protein for peptide substrates.

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