Characterization of a Lidless Form of the Molecular Chaperone DnaK

DELETION OF THE LID INCREASES PEPTIDE ON- AND OFF-RATE CONSTANTS*

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The C-terminal, polypeptide binding domain of the 70-kDa molecular chaperone DnaK is composed of a unique lidlike subdomain that appears to hinder steric access to the peptide binding site. We have expressed, purified, and characterized a lidless form of DnaK to test the influence of the lid on the ATPase activity, on interdomain communication, and on the kinetics of peptide binding. The principal findings are that loss of the lid creates an activated form of DnaK which is not equivalent to ATP-bound DnaK. For example, at 25 °C the NR peptide (NRLLLTG) dissociates from the ADP and ATP states of DnaK with observed off-rate constants of 0.001 and 4.8 s⁻¹, respectively. In contrast, for DnaK that lacks most of the helical lid, residues 518–638, the NR peptide dissociates with observed off-rate constants of 0.1 and 188 s⁻¹. These results show that the loss of the lid does not interfere with interdomain communication, that the β-sandwich peptide binding domain can exist in two discrete conformations, and that the lid functions to increase the lifetime of a DnaK-peptide complex. We discuss several mechanisms to explain how the lid affects the lifetime of a DnaK-peptide complex.

DnaK, the 70-kDa molecular chaperone expressed by Escherichia coli, functions in protein folding, assembly, transport, and proteolysis in an ATP-dependent activity cycle that is regulated by the two cochaperones, GrpE and DnaJ (1–4). DnaK also functions in more specialized activities such as bacteriophage λ DNA replication (5, 6). In all of these processes, DnaK is thought to interact transiently with unraveled segments of partially unfolded or denatured protein substrates. Studies have shown that the reversible switching from a high affinity conformation, which binds substrate tightly, to a low affinity conformation, which binds substrate weakly, is the hallmark of DnaK activity (7–11). Such a mechanism is shown in Scheme 1, where ADP-DnaK-P and ATP-DnaK* are the high and low affinity states, respectively, and E and J are GrpE and DnaJ, respectively. In this report, we examine the role of a specific subdomain of DnaK on this conformational switch.

DnaK is composed of two functional domains: residues 1–387 comprise the N-terminal ATPase domain, and residues 388–638 comprise the C-terminal polypeptide binding domain. The three-dimensional structure of the ATPase domain of bovine brain Hsc70, a 70-kDa chaperone, is a bilobed structure in which a nucleotide molecule sits at the base of a cleft formed by the two lobes (12). The three-dimensional structure of a fragment of the C-terminal polypeptide binding domain comprising residues 387–601 consists of a β-sandwich subdomain that is followed by an α-helical subdomain that acts like a lid over the β-sandwich subdomain (13, 14) (Fig. 1). The bound peptide contacts the β-sandwich but not the lid.

The molecular structure depicted in Fig. 1, with the lid subdomain blocking entry to and exit from the peptide binding site, has been suggested to be the high affinity conformation of the C-terminal domain of DnaK (14). ATP binding to the N-terminal domain induces a global conformational change in DnaK (7) which is thought to displace the lid from the top of the β-sandwich subdomain, creating the low affinity form of the protein. To gain insight into the function of the lid, slightly different lidless forms of DnaK and eukaryotic Hsp70s have been engineered (9, 15–18). What has emerged from these studies is that loss of the lid does not interfere with interdomain coupling (15), and loss of the lid results in a 2–20-fold increase in $K_d$ values for peptide interaction with the ADP-bound state of lidless DnaK (17, 18). The lidless protein reversibly oligomerizes (16). The β-sandwich subdomain can exist in a closed conformation (17). Lidless DnaK(2–538) does not function in an in vitro folding assay (18), whereas DnaK(1–507) supports λ phage DNA replication in vivo, albeit at reduced activity relative to the wild type protein (17).

The hypothesis tested in this study was that removal of the DnaK lid should produce a constitutive low affinity state of the protein. In other words, if the lid enables high affinity peptide binding to DnaK, then deletion of the lid should produce a state that rapidly binds and rapidly releases peptide, even in the presence of ADP. Such experiments are important because the kinetics of peptide interactions with lidless DnaK is unexplored. To this end, we have expressed, purified, and characterized a lidless variant of DnaK which contains an N-terminal glutathione S-transferase (GST)¹ tag. Deletion of these residues (see the arrow in Fig. 1) completely eliminates four of the five helices that make up the lid. Because GST domains dimerize (19, 20), the GST tag was removed by thrombin cleavage prior to characterization. In agreement with two earlier studies (17, 18), we show here that our GST-cleaved DnaK(1–517) is not comparable to the ATP-bound low affinity state of DnaK. In-

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¹ The abbreviations used are: GST, glutathione S-transferase; clDnaK, GST-cleaved DnaK; HPLC, high performance liquid chromatography; NR, a synthetic peptide (NRLLTTG); Cro, synthetic peptide representing residues 1–12 of the Cro repressor protein (MQERITLK-DYAM); wt, wild type; INR, α-N, dansyl-NR peptide.
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Wild Type DnaK and Peptides—All reagents were purchased from Sigma Chemicals, unless otherwise noted. DnaK was purified as described (21, 22) and maintained in sample buffer consisting of HEPES, 50 mM KCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol at pH 7.0. Nucleotide-free DnaK was prepared by an exhaustive 4-day dialysis (22, 23). Dialysis removes more than 95% of bound nucleotide and is as effective as the chemical method of Gao et al. (24). SDS-polyacrylamide gel electrophoresis demonstrated that the wtDnaK preparations, as well as the DnaK variant preparations described below, were ≥95% pure. Unless stated otherwise, protein concentrations were based on quantitative amino acid composition analyses, which were conducted at the University of Nebraska (Protein Structure Core Facility, Omaha).

Peptides were synthesized by Genemed Synthesis Inc. (South San Francisco), purified to >95% by HPLC, and peptide mass was verified by electrospray mass spectroscopy. The NR (25) peptide was dansylated at its N terminus by reacting the peptide for 1 h with a 2-fold molar excess of dansyl chloride (Molecular Probes) in the presence of an equimolar amount of the organic base diethylisopropylamine in aqueous solution. Unreacted label was removed by passing the reaction mixture of 50 mM EDTA, 4 mM ATP, and 4 mM ADP. Aliquots were removed periodically, and the reaction was stopped by mixing with a solution of 50 mM EDTA, 4 mM ATP, and 4 mM ADP. Aliquots were removed periodically, and the reaction was stopped by mixing with a solution of 50 mM EDTA, 4 mM ATP, and 4 mM ADP. Aliquots were concentrated.

ATPase Activity Assay—This assay was conducted as described previously (21) with minor modifications. Briefly, the assay mixture (60 μl) was composed of the following reagents: 1–4 μM DnaK, 0–600 μM peptide, 10 μM [2,8-3H]ATP (36 Ci/mmol) (NEN Life Science Products, Boston, MA), 1 Ci = 3.7 × 10¹⁰ Bq), and 70 μM ATP in a HEPES buffer (40 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.6). Reactions were conducted at 25 °C. Aliquots were removed periodically, and the reaction was stopped by mixing with a solution of 50 mM EDTA, 4 mM ATP, and 4 mM ADP. Aliquots were concentrated.

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free [3H]Cro peptide is retained on the column. Experiments were conducted as follows. Prior to spinning samples to separate DnaK-peptide complexes from free peptide, each spin column was equilibrated in the HEPES sample buffer. Samples containing 4 μM DnaK, 20 μM [3H]Cro, and 1 mM ADP or ATP were then incubated for 1 h at 25 °C. An aliquot of each reaction mixture (50 μl) was loaded on the spin column, and the columns were then spun at 1,000 × g for 4 min. Two assays were conducted on the liquid (nucleotide-DnaK-[3H]Cro complexes) run-through. One aliquot (30 μl) was assayed for tritium content by liquid scintillation; the other aliquot (20 μl) was assayed for protein concentration using a Bio-Rad protein assay kit. The protein determination step after each spin column run enabled us to determine whether any DnaK was retained by the column. This measurement increased the precision and accuracy of the method. Control experiments showed that no appreciable free tritiated peptide passes through the spin column.

Fluorescence Measurements—A Photon Technology Inc. (South Brunswick, NJ) StrobeMaster lifetime spectrometer with an SE-900 steady-state fluorescence option was used to obtain both steady-state and kinetic data. The steady-state measurements were obtained using a 75-watt xenon arc lamp excitation source in conjunction with photon counting detection (PTI model 710). The excitation wavelength was 295 nm, and the emission was scanned over the wavelength range of 300–400 nm. The excitation and emission slits were typically 2 and 5 nm, respectively. Emission spectra were corrected for the instrumental response. For each type of measurement, samples were maintained in a quartz cuvette (1-cm path length) with constant stirring and temperature control via an external circulating heating/cooling bath (ΔT = ± 0.2 °C). Sample temperature was verified using a hand-held thermometer placed directly into the sample. Kinetic experiments were conducted in time base mode.

Rapid kinetics experiments were conducted with an Applied Photophysics (Leatherhead, U. K.) SX-18MV stopped-flow fluorescence spectrometer, which has a 1.5-ms dead time. The excitation wavelength for the dansyl-labeled peptide was 335 nm, and emission was collected using a 399 cut-off filter. Additional details about this instrument may be found in Ref. 22. Kinetic traces are the average of four to seven individual runs.

Data Analysis and Reproducibility of Measurements—Error bars in the ATPase and peptide binding experiments represent the S.E. of the mean of duplicate or triplicate determinations. Stopped-flow data were analyzed using a curve fitting program supplied with the Applied Photophysics instrument; the program uses a Marquardt algorithm based on the program Curfit described by Bevington (28). Stopped-flow experiments were repeated several times over the course of several weeks. The program KaleidaGraph (Synergy Software, Reading, PA) was used for the numerical fitting of the plots of k_on versus [protein]. Reported errors in the slope and intercept of these plots are standard errors, computed by this program.

RESULTS

Wild type DnaK and two variants were studied. (i) The primary form of lidless DnaK used in this report was expressed with an N-terminal GST tag that was removed by treatment with thrombin prior to characterization. N-terminal sequence analysis revealed that 10 residues, GSPEFPGRLE, remain attached to the N terminus of DnaK after cleavage of the GST tag. GSPEFPGRLE-DnaK(1–517) is referred to below as clDnaK(1–517). (ii) To control for the effect, if any, of this 10-amino acid prepiece a GST fusion of wtDnaK was also expressed; cleavage of the tag leads to GSPEFPGRLE-DnaK, and this form of the protein is referred to as clDnaKwt.

ATPase Activity—The steady-state ATPase activities of wild type, clDnaK(1–517), and clDnaKwt are compared in Fig. 2. In the absence of peptide, the steady-state ATPase activity of wtDnaK was 0.001 min⁻¹, and added peptide increased this value 5-fold to 0.11 ± 0.01 min⁻¹. In contrast, in the absence of peptide clDnaK(1–517) hydrolyzes ATP (0.05 ± 0.015 min⁻¹) about two times faster than wtDnaK, and, in agreement with Pellecchia and co-workers (17), added peptide produces a 2-fold stimulation of the ATPase activity of lidless DnaK. As a control, we determined the steady-state ATPase activity of clDnaKwt. The ATPase values with and without peptide are almost identical to those found for clDnaK(1–517).

On the basis of these results we conclude that clDnaK(1–517) is fully capable of binding peptide because added peptide stimulates its ATPase activity, and the 10-amino acid prepiece stimulates the endogenous ATPase activity of DnaK by a factor of 2.

Peptide Binding—The effect of nucleotide on equilibrium peptide binding to wild type, clDnaK(1–517), and clDnaKwt is shown in Fig. 3. A spin column assay was used instead of size exclusion column because the former assay enables the separation of relatively short lived DnaK-peptide complexes from free peptide, whereas the latter method requires relatively long lived complexes (t_1/2 > 15 min). The assay was conducted as described under “Experimental Procedures.” A tritiated Cro peptide ([3H]MQERITLKDYAM) was used.

Wild type DnaK binds the [3H]Cro peptide in a nucleotide-dependent fashion, with high affinity binding and reduced or low affinity binding in the presence of ADP and ATP (Fig. 3), respectively. By comparison, clDnaK(1–517) binds the [3H]Cro peptide with about one-half to one-fourth the affinity as wtDnaK in the presence of ADP. However, similar to wtDnaK, added ATP reduces the amount of peptide binding to the lidless protein. To control for the presence of the prepiece, [3H]Cro peptide binding to clDnaKwt was also examined. This form of DnaK displays high and low affinity binding in the presence of ADP and ATP, respectively. One difference between clDnaK and wtDnaK is that there is about twice the amount of peptide binding in the presence of ATP to the cleaved form of wild type. The reduced amount of [3H]Cro peptide binding to clDnaK(1–517), coupled with the ability of ATP to reduce further the amount of binding, agrees with earlier studies (17, 18).

Kinetics of DnaK-Peptide Complex Formation/Dissociation—

The effect of the loss of the lid on the kinetics of DnaK-peptide complex formation and dissociation was determined using a dansyl-labeled form of the NR peptide. A fluorescence spectrometer in time-based mode was used for experiments conducted on wtDnaK and clDnaKwt, and a stopped-flow instrument was used for experiments conducted on clDnaK(1–517).

Fig. 4A compares INR dissociation from wild type, clDnaKwt, and clDnaK(1–517) in the presence of ADP. Over a time base of 2,000–3,000 s for dissociation from wtDnaK and clDnaKwt and a time base of 100 s for clDnaK(1–517), INR dissociation follows single exponential kinetics. The dissociation of the NR peptide from preformed wtDnaK-INR complexes occurs with an apparent first-order rate constant, k_off, equal to 1.0 (± 0.1) × 10⁻³ s⁻¹. In contrast, the dissociation of the NR peptide from preformed clDnaK(1–517)-NR complexes is 100 times faster, occurring with an apparent k_off equal to 1.1 (± 0.2) × 10⁻¹ s⁻¹. (An expanded scale version of this dissociation trace is shown in the
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FIG. 3. Equilibrium peptide binding to DnaK variants. A spin column assay was used to measure equilibrium peptide binding to DnaK and its various forms (wtDnaK, cDnaK(1–517), and cDnaKwt). For details regarding the spin column assay, see “Experimental Procedures.” Conditions: [protein] = 4 μM; [3H]Cro = 20 μM; temperature = 25 °C.

FIG. 4. Kinetics of DnaK-peptide complex dissociation. Panel A, comparison of fNR dissociation from wild type, cDnaKwt, and cDnaK(1–517) in the presence of ADP. Experiments were initiated by mixing preformed ADP-wtDnaK-fNR complexes with excess unlabeled fNR plus excess ADP. Each dissociation trace was fit to a single exponential function (solid line). wtDnaK → wtDnaK + fNR, k_{off} = 0.00099 s^{-1}; cDnaKwt → cDnaKwt + fNR, k_{off} = 0.00083 s^{-1}; and cDnaK(1–517) → cDnaK(1–517) + fNR, k_{off} = 0.073 s^{-1}. The inset shows an expanded scale trace for dissociation from cDnaK(1–517). Concentrations after mixing: [protein] = 1–2 μM; [fNR] = 30–60 μM; [ADP] = 1 mM. Temperature = 25 °C. Panel B, comparison of fNR dissociation from wild type, cDnaKwt, and cDnaK(1–517) in the presence of ATP. Each dissociation trace was fit to a single exponential function (solid line). wtDnaK-fNR → wtDnaK + fNR, k_{off} = 4.8 s^{-1}; cDnaKwt-fNR → cDnaKwt + fNR, k_{off} = 4.1 s^{-1}; and cDnaK(1–517)-fNR → cDnaK(1–517) + fNR, k_{off} = 0.13 s^{-1}. The inset shows an expanded scale trace for fNR dissociation from cDnaK(1–517). Concentrations after mixing: [protein] = 1–2 μM; [fNR] = 30–60 μM; [ATP] = 1 mM. Temperature = 25 °C.

Inset.) As a control, the dissociation of the fNR peptide from cDnaKwt, which, like cDnaK(1–517), possesses a 10-amino acid, N-terminal prepiece, was examined. The fNR peptide dissociates from cDnaKwt with k_{off} = 8.7 (± 0.5) × 10^{-4} s^{-1}, which demonstrates that the 10-amino acid prepiece does not affect the rate of fNR dissociation from DnaK. The dissociation experiments show that loss of the lid increases the rate of peptide release from ADP-DnaK by 2 orders of magnitude.

FIG. 5. Kinetics of DnaK-peptide complex formation. Panel A, kinetics of ADP-cDnaK(1–517)-fNR complex formation. The reagents in the two stopped-flow syringes are indicated in brackets: [cDnaK(1–517)] + ADP + [fNR]. Concentrations after mixing: [fNR] = 1.0 μM, and [cDnaK(1–517)] = 15 (curve a), 10 (curve b), and 5 (curve c) μM; 1 mM ADP. All curves follow double exponential kinetics. Residuals to the fit of curve a to a single and double exponential functions are shown above the panel containing the three curves. The double exponential function, F(t) = 0.104 exp(-0.326t) + 0.169 exp(-0.063t) + 0.342, yielded the best fit. Panel B, plots of k_{obs1} versus [cDnaK(1–517)]. Panel C, kinetics of ADP-wtDnaK-fNR complex formation. The reagents in the two stopped-flow syringes are indicated in brackets: [wtDnaK] + ADP + [fNR]. Concentrations after mixing: [fNR] = 0.2 μM, and [wtDnaK] = 8 (curve a), 4 (curve b), and 1 (curve c) μM; 1 mM ADP. Curves follow single exponential kinetics (solid lines). Residuals from the fit of curve a are shown in the panel above the three curves. Panel D, plot of k_{obs2} versus [cDnaKwt]. The solid line is the least squares fit of the data sets to the equation k_{obs2} = k_{sz} [cDnaK(1–517)] + k_{sz} (solid line). Concentrations after mixing: [protein] = 1–2 μM; [fNR] = 1–2 μM; [ADP] = 1 mM. The fNR peptide binds to ADP-bound, lidless DnaK with an expanded scale trace for fNR dissociation from cDnaK(1–517) in the presence of ATP. Each dissociation trace was fit to a single exponential function, wtDnaK → wtDnaK + fNR, k_{off} = 0.00099 s^{-1}; cDnaKwt → cDnaKwt + fNR, k_{off} = 0.00083 s^{-1}; and cDnaK(1–517) → cDnaK(1–517) + fNR, k_{off} = 0.073 s^{-1}. The inset shows an expanded scale trace for fNR dissociation from cDnaK(1–517). The least squares fit to the equation k_{obs1} = k_{sz} [cDnaK(1–517)] + k_{sz} (solid line) yields k_{sz} = 11.00 ± 2.000 m^2 s^{-1} and k_{sz} = 0.13 ± 0.04 s^{-1}. The asymptote of the curve equals 0.06–0.07 s^{-1}. Error bars represent the S.E. of duplicates. Panel C, kinetics of ADP-wtDnaK-fNR complex formation. The reagents in the two stopped-flow syringes are indicated in brackets: [wtDnaK] + ADP + [fNR]. Concentrations after mixing: [fNR] = 0.2 μM, and [wtDnaK] = 8 (curve a), 4 (curve b), and 1 (curve c) μM; 1 mM ADP. Curves follow single exponential kinetics (solid lines). Residuals from the fit of curve a are shown in the panel above the three curves. Panel D, plot of k_{obs2} versus [cDnaKwt]. The solid line is the least squares fit of the data sets to the equation k_{obs2} = k_{sz} [cDnaK(1–517)] + k_{sz} (solid line). Concentrations after mixing: [protein] = 1–2 μM; [fNR] = 1–2 μM; [ADP] = 1 mM. Temperature = 25 °C.
the following sequential two-step mechanism

\[
P + \text{ADP-lidless} \rightleftharpoons (\text{ADP-lidless}P) \rightleftharpoons (\text{ADP-lidless}P)_1 \rightleftharpoons (\text{ADP-lidless}P)_2
\]

**REACTION 1**

where \((\text{ADP-lidless}P)_1\) and \((\text{ADP-lidless}P)_2\) denote intermediate and terminal complexes of clDnaK(1–517). For Reaction 1, when the bimolecular reaction \(P + \text{ADP-lidless} \rightleftharpoons (\text{ADP-lidless}P)_1\) is faster than the unimolecular reaction \((\text{ADP-lidless}P)_1 \rightleftharpoons (\text{ADP-lidless}P)_2\). \(k_{\text{obs1}}\) exhibits a linear dependence on the concentration of lidless DnaK, whereas \(k_{\text{obs2}}\) exhibits a hyperbolic dependence on the concentration of the lidless protein (29). The plot of \(k_{\text{obs1}}\) versus \([\text{clDnaK(1–517)}]\) (see Fig. 5B) is linear; the slope and intercept yield apparent on- and off-rate constants of 11,000 (± 2,000) \(\text{s}^{-1}\) and 0.13 (± 0.04) \(\text{s}^{-1}\), respectively. This latter value agrees, within the experimental error, with the value of \(k_{\text{off1}}\) determined from the direct dissociation experiment (Table I). The plot of \(k_{\text{obs2}}\) versus \([\text{clDnaK(1–517)}]\) (see Fig. 5B) shows a weak dependence of \(k_{\text{obs}}\) on \([\text{clDnaK(1–517)}]\), and the asymptote of the plot, \(k_{\text{off1}} + k_{\text{off2}}\), equals 0.06–0.07 \(\text{s}^{-1}\).

For comparison, the kinetics of complex formation between the fNR peptide and wtDnaK in the presence of excess ADP was investigated. The reactions were carried out at a fixed concentration of the fNR peptide (0.2 \(\mu\text{M}\)) while the concentration of wtDnaK was varied (2–10 \(\mu\text{M}\)). Over this range of protein concentrations, the fNR peptide binds to ADP-wtDnaK in one phase (\(F(\tau) = \Delta F \exp[-k_{\text{obs}} \tau] + F_s\)) (Fig. 5C), consistent with complex formation according to Reaction 2.

\[
P + \text{ADP-wtDnaK} \rightleftharpoons \text{ADP-wtDnaKP}
\]

**REACTION 2**

Assuming complex formation according to Reaction 2, a plot of \(k_{\text{obs}}\) versus [wtDnaK] should be linear, with slope and intercept equal to the apparent on-rate (\(k_{\text{on}}\)) and off-rate (\(k_{\text{off}}\)) constants, respectively. The plot of \(k_{\text{obs}}\) versus [wtDnaK] in Fig. 5D is linear and yields apparent on- and off-rate constants of 910 (± 40) \(\text{s}^{-1}\) and 0.0019 (± 0.0002) \(\text{s}^{-1}\), respectively. This latter value is similar to the apparent \(k_{\text{off}}\) value determined from the direct dissociation experiment (\(k_{\text{off}} = 0.0010 \pm 0.0001 \text{s}^{-1}\)) (Table I). These combined kinetic experiments show that deletion of the lid increases the magnitudes of the apparent on- and off-rate constants (\(k_{\text{on}}\) and \(k_{\text{off}}\)) by 12- and 100-fold, respectively.

When complex formation reactions were carried out at a larger fixed concentration of the fNR peptide (1.0 \(\mu\text{M}\)) while the concentration of wtDnaK was varied over a larger range (4–20 \(\mu\text{M}\)) there was a rapid (\(k_{\text{obs}} = 2 \text{s}^{-1}\)) initial increase in fluorescence which constituted 5% of the total signal change. Based on this observation, it is likely that the reaction between wtDnaK and the fNR peptide is also biphasic, but because of the small amplitude we could not characterize this phase.

**DISCUSSION**

Striking differences were found in the kinetic constants for the interactions of the NR peptide with wtDnaK and lidless DnaK (Table I). Differences in off-rate constants between wild type and lidless DnaK are illustrated in Reactions 3–6. Reactions 4 and 5 compare peptide dissociation from wild type and clDnaK(1–517) in the presence of ADP, respectively; Reactions 5 and 6 compare peptide dissociation from wild type and clDnaK(1–517) in the presence of ATP, respectively.

$$0.001 \text{s}^{-1}$$

$$\text{ADP-wtDnaK-Q} \longrightarrow \text{ADP-wtDnaK + fNR}$$

**REACTION 3**

$$0.1 \text{s}^{-1}$$

$$\text{ADP-clDnaK(1–517)+QR} \longrightarrow \text{ADP-clDnaK(1–517) + fNR}$$

**REACTION 4**

$$5 \text{s}^{-1}$$

$$(\text{ATP + wtDnaK-Q}) \longrightarrow \text{ATP-wtDnaK* + fNR}$$

**REACTION 5**

$$188 \text{s}^{-1}$$

$$(\text{ATP + clDnaK(1–517)+Q}) \longrightarrow \text{ATP-clDnaK(1–517)+* + fNR}$$

**REACTION 6**

The above results show that loss of the lid produces an activated form of DnaK in which the rate constants for peptide dissociation are increased substantially compared with wild type. For example, in the presence of ADP the deletion of the lid increases \(k_{\text{off}}\) by a factor of 100 (0.001 → 0.1 \text{s}^{-1}), whereas in the presence of ATP the deletion of the lid increases \(k_{\text{off}}\) by a factor of 37.6 (5 → 188 \text{s}^{-1}). Further, that the \(k_{\text{off}}\) value for NR peptide dissociation from clDnaK(1–517) is so nucleotide-dependent, increasing from 0.1 to 188 \text{s}^{-1} when ADP is replaced with ATP shows that the β-sandwich subdomain can exist in two distinct conformations, in agreement with earlier studies (17, 18). The combined results show that the lid acts like a brake on the rate of ATP-induced peptide dissociation and that the lid and the β-sandwich facilitate peptide binding to DnaK.

One of the findings in this report is that the presence of DnaK lid increases the lifetime of a Dnakeptide complex. For example, in the presence of ATP the lifetime, \(\tau\), of an ATP-DnaK*QNR complex is 140 ms (ln 2/5 \text{s}^{-1}), whereas deletion of the lid decreases the lifetime to 3.7 ms (ln 2/188 \text{s}^{-1}). To explain the effect of the lid on the lifetime of the ATP-DnaK-peptide complex, below we discuss several variations of a two-step reaction mechanism that involves sequential conformational changes in the lid and β-sandwich subdomains. For simplicity, only forward reactions, i.e. closed-to-open transitions in the β-sandwich (β-c-o) and lid
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In Reaction 9, for wild type protein, ATP binding triggers the \( \beta \)-sandwich to open in the first step \( (k_{b\rightarrow a} = 5 \text{ s}^{-1}) \), and the lid opens in the second step \( (k_{b\rightarrow c} > 5 \text{ s}^{-1}) \). We propose that deletion of the lid alters the structure of the \( \beta \)-sandwich \( (\beta') \) which results in an increase in the magnitude of the rate constant for peptide dissociation to \( 188 \text{ s}^{-1} \) (Reaction 10). Because the \( \beta \)-sandwich and lid are connected by a latch, a network of hydrogen bonds, and a salt bridge \( (14, 18) \), it is very likely that deletion of the lid affects the structure and reactivity of the \( \beta \)-sandwich subdomain. It will be challenging to design experiments to distinguish among these various mechanisms.

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(\( k_{b\rightarrow a} \)), are depicted.

If the \( \beta \)-sandwich and lid subdomains are uncoupled in that deletion of the lid does not affect the rate constants for the \( \beta_{c\rightarrow b} \) or \( \beta_{c\rightarrow a} \) transitions, then our results are consistent with Reaction 7, where ATP binding triggers conformational changes first in the \( \beta \)-sandwich and then in the lid

\[
188 \text{ s}^{-1} \quad \text{Reaction 7}
\]

\[
5 \text{ s}^{-1} \quad \text{ATP + E} \rightarrow \text{ATP}E \quad \text{ATP} \rightarrow \text{ATP}E + P
\]

where \( E \) is the high affinity wtDnaK-peptide complex in which both the \( \beta \)-sandwich and lid are closed; \( \text{ATP}E \) is an intermediate in which the \( \beta \)-sandwich and lid are open and closed, respectively; and \( \text{ATP}E \) is the low affinity state in which both the \( \beta \)-sandwich and lid subdomains are open. Note that the second step in Reaction 7, lid opening \( (k_{b\rightarrow a} = 5 \text{ s}^{-1}) \), is the rate-limiting step for peptide dissociation peptide. Upon deletion of the lid peptide dissociation is governed by the \( \beta_{c\rightarrow a} \) transition, therefore the maximal rate of peptide dissociation jumps from 5 to 188 s\(^{-1}\). Because at present we cannot determine the sequence of the conformational changes upon ATP binding to DnaK, it must be noted that our results are also consistent with Reaction 8, where ATP binding triggers conformational changes first in the lid and then in the \( \beta \)-sandwich.

\[
188 \text{ s}^{-1} \quad \text{Reaction 8}
\]

\[
5 \text{ s}^{-1} \quad \text{ATP + E} \rightarrow \text{ATP}E \quad \text{ATP} \rightarrow \text{ATP}E + P
\]

In the above reaction, the first step, lid opening \( (k_{b\rightarrow a} = 5 \text{ s}^{-1}) \), is the rate-limiting step for peptide dissociation peptide from wtDnaK. Upon deletion of the lid peptide dissociation becomes governed by the \( \beta_{c\rightarrow a} \) transition, therefore the maximal rate of peptide dissociation jumps from 5 to 188 s\(^{-1}\).

Alternatively, suppose the \( \beta \)-sandwich and lid subdomains are coupled in that deletion of the lid affects the magnitudes of the rate constants for the \( \beta_{c\rightarrow a} \) or \( \beta_{c\rightarrow a} \) transitions. In this case, the effect of removal of the lid is depicted in Reactions 9 and 10.

\[
188 \text{ s}^{-1} \quad \text{Reaction 9}
\]

\[
5 \text{ s}^{-1} \quad \text{ATP + E} \rightarrow \text{ATP}E \quad \text{ATP} \rightarrow \text{ATP}E + P
\]

In Reaction 9, for wild type protein, ATP binding triggers the \( \beta \)-sandwich to open in the first step \( (k_{b\rightarrow a} = 5 \text{ s}^{-1}) \), and the lid opens in the second step \( (k_{b\rightarrow c} > 5 \text{ s}^{-1}) \). We propose that deletion of the lid alters the structure of the \( \beta \)-sandwich \( (\beta') \) which results in an increase in the magnitude of the rate constant for peptide dissociation to \( 188 \text{ s}^{-1} \) (Reaction 10). Because the \( \beta \)-sandwich and lid are connected by a latch, a network of hydrogen bonds, and a salt bridge \( (14, 18) \), it is very likely that deletion of the lid affects the structure and reactivity of the \( \beta \)-sandwich subdomain. It will be challenging to design experiments to distinguish among these various mechanisms.

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Characterization of a Lidless Form of the Molecular Chaperone DnaK: DELETION OF THE LID INCREASES PEPTIDE ON- AND OFF-RATE CONSTANTS
Greg Buczynski, Sergey V. Slepenkov, Michael G. Sehorn and Stephan N. Witt

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