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Energetics of Nucleotide-Induced DnaK Conformational States†

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ABSTRACT: Hsp70 chaperones are molecular switches that use the free energy of ATP binding and hydrolysis to modulate their affinity for protein substrates and, most likely, to remodel non-native interactions allowing proper substrate folding. By means of isothermal titration calorimetry, we have measured the thermodynamics of ATP and ADP binding to (i) wild-type DnaK, the main bacterial Hsp70; (ii) two single-point mutants, $DnaK_{T199A}$, which lacks ATPase activity but maintains conformational changes similar to those observed in the wild-type protein, and $DnaK_{R151A}$, defective in interdomain communication; and iii) two deletion mutants, the isolated nucleotide binding domain (K-NBD) and a ΔLid construct [DnaK(1−507)]. At 25 °C, ATP binding to DnaK results in a fast endothermic and a slow exothermic process due to ATP hydrolysis. We demonstrate that the endothermic event is due to the allosteric coupling between ATP binding to the nucleotide binding domain and the conformational rearrangement of the substrate binding domain. The interpretation of our data is compatible with domain docking upon ATP binding and shows that this conformational change carries an energy penalty of ca. 1 kcal/mol. The conformational energy stored in the ATP-bound DnaK state, together with the free energy of ATP hydrolysis, can be used in remodeling bound substrates.

Essential cellular processes such as folding of nascent protein chains, protection from several stress types, translocation of protein across membranes, and solubilization of protein aggregates are facilitated by Hsp70 chaperones (*1*−*3*). Hsp70 proteins contain two domains (*4*): a conserved N-terminal nucleotide binding domain (NBD)[1] able to bind and slowly hydrolyze ATP (*5*) and a C-terminal substrate binding domain (SBD) that interacts with short hydrophobic peptide stretches of partially (un)folded proteins. A highly conserved hydrophobic linker, essential for chaperone activity, connects both domains (*6*, *7*). The function of Hsp70 chaperones is based on a complex interdomain allosteric communication, which promotes protein cycling between two main conformations: an ATP-bound conformation (T) in which an open SBD accepts peptide substrates and an ADP-bound conformation (R) with a closed SBD that tightly binds polypeptides. Hsp40 proteins and nucleotide exchange factors stimulate these conformational transitions (*8*, *9*).

Several biochemical and structural studies over the past decade have formulated the current understanding of the Hsp70 allosteric mechanism (*6*, *10*−*18*). In the T conformation, the linker interacts with the NBD and promotes tethering of both domains (*17*). Domain docking stabilizes some regions of the SBD, while others, mainly those forming the substrate binding pocket, become dynamic (*17*). Substrate binding stimulates ATP hydrolysis and conversion to the R conformation in which the NBD and SBD, connected by a flexible linker, separate and behave independently (*17*, *18*). The structure of full-length DnaK in the R state has recently been published by Zuiderweg and co-workers (*18*), while that of the T state remains elusive. Mutagenesis and proton−deuterium exchange studies suggest that subdomain IA of the NBD may contact loops L2,3 and L6,7 in the β-sandwich subdomain of the SBD (*10*, *15*, *16*). Although the helical lid is not strictly required for the transmission of at least part of the allosteric signal (*19*), this subdomain may also contact the NBD (*14*) and is essential for the stabilization of the interdomain interface at elevated temperatures (*20*).

The aim of this study is to characterize the thermodynamics of nucleotide binding to DnaK, the main bacterial Hsp70. Our results indicate that ATP and ADP binding to DnaK differ energetically, while they bind similarly to the isolated NBD, suggesting that the nucleotide-induced allosteric conformational change in the SBD modifies the thermodynamics of binding. With regard to the conformational equilibrium between the T and R conformations, our data show that conversion to the T state is unfavored and carries an energy penalty. The interpretation of our data agrees with the domain docking model (*17*) and gives an estimation of the conformational energy that the chaperone might provide to remodel peptide substrates.

MATERIALS AND METHODS

Cloning and Protein Purification. Expression plasmids for $DnaK_{T199A}$ and $DnaK_{R151A}$ were a kind gift from M. Mayer. Deletion mutants $K\text{-}NBD_{T199A}$ and $DnaK(1-507)_{T199A}$ were

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[1]Abbreviations: NBD, nucleotide binding domain; SBD, substrate binding domain; ITC, isothermal titration calorimetry; ΔLid, DnaK-(1−507) construct; wt, wild-type.

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amplified by PCR using as a template the $DnaK_{T199A}$ vector and cloned as described previously (*14*). Proteins were purified following a standard protocol (*14*).

ITC Measurements. Calorimetric measurements were performed in a VP-ITC microcalorimeter (MicroCal LLC, Northampton, MA). Proteins were dialyzed against 40 mM Tris-HCl (pH 7.4), 50 mM KCl, and 10 mM $MgCl_2$, and their concentration was determined using an extinction coefficient (ε_{280}) of $15.8 \times 10^3\ M^{-1}\ cm^{-1}$. Nucleotides (Sigma) were freshly dissolved in dialysis buffer prior to ITC measurements. The single-injection experiments were performed using a protein concentration of ~20 μM and injection of 10 μL of a 2.8−2.9 mM ATP solution into the calorimetric cell. Typical titration experiments were performed with proteins carrying the single-point mutation T199A (10−15 μM). Consecutive injections of a nucleotide solution (~60 μM) were conducted after sample equilibration. The same ATP and ADP solutions were injected into dialysis buffer to estimate the dilution enthalpy that was used to correct the binding enthalpy. Binding isotherms were analyzed using a single-site binding model to determine the apparent thermodynamic parameters. The temperature dependence of these parameters was analyzed by nonlinear least-squares fitting to the model described in the Appendix using Origin version 7.0 (OriginLab Corp., Northampton, MA).

RESULTS

ATP Binding to DnaK and $DnaK_{T199A}$ (single injection). Although the intrinsic ATPase activity of DnaK in the absence of substrates and/or cochaperones is low, the strong exothermic nature of the process hampers the analysis of ATP titraton experiments. Therefore, to observe, at least qualitatively, the energetics of ATP binding to wt DnaK, we set up an experiment in which all DnaK molecules were saturated with nucleotide in a "single ligand injection" (Figure 1A). Such measurement resulted in a fast endothermic signal that overlapped with a slow exothermic one. These events can be tentatively assigned to ATP binding and hydrolysis, respectively, in good agreement with the published time evolution of the intrinsic fluorescence and difference infrared spectroscopy signals in the presence of ATP (*20*, *21*). To validate this assignment, we first avoided the hydrolysis contribution by using a mutant ($DnaK_{T199A}$) unable to hydrolyze ATP and as sensitive as the wt protein to nucleotide-induced conformational changes (*11*, *20*). As shown in Figure 1B, only the endothermic event was observed with this mutant. When the same experiment was conducted with $DnaK_{R151A}$, a mutant defective in allosteric communication (*15*), the endothermic signal was abolished and only an exothermic event due to nucleotide binding and hydrolysis was detected (Figure 1C). Taken together, these data validate the assignment of the endothermic event to the allosteric conformational change coupled to the nucleotide binding reaction.

We first tried to quantify the thermodynamics of ATP binding to wt DnaK with titration experiments using nonhydrolyzable ATP analogues (AMP-PNP and ATPγS). However, none of the analogues exerted the same effect as ATP, as previously reported (*22*), their binding thermodynamic parameters resembling those observed for ADP binding to $DnaK_{T199A}$ (Table 1). Failure of these analogues to induce an ATP-like conformation might be due to the missing specific interactions that the $\beta-\gamma$ oxygen bond and γ-phosphate establish with the protein and magnesium ion (*22*).

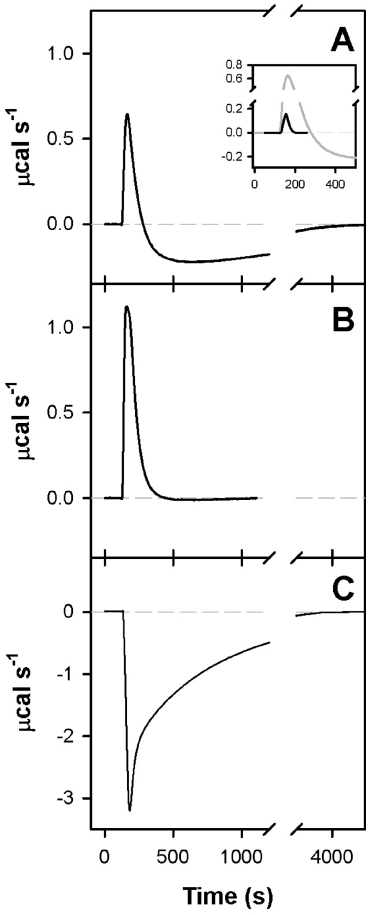

FIGURE 1: ATP binding to DnaK, $DnaK_{T199A}$, and $DnaK_{R151A}$ (single-injection experiments). Calorimetric traces of a single injection of a stoichiometric concentration of ATP into a solution of DnaK (A), $DnaK_{T199A}$ (B), or $DnaK_{R151A}$ (C). The protein concentration was ca. 20 μM. The experiments were performed in 40 mM Tris-HCl (pH 7.4), 50 mM KCl, and 10 mM $MgCl_2$ at 25 °C. The inset shows the injection of ATP in buffer under the same experimental conditions.

Nucleotide Binding to $DnaK_{T199A}$ and K-NBD_{T199A}. ATP and ADP titration experiments were performed with full-length $DnaK_{T199A}$ and its isolated nucleotide binding domain (K-NBD_{T199A}). As expected, ATP binding to $DnaK_{T199A}$ at 25 °C induced a positive enthalpy change, indicating an endothermic reaction (Figure 2A, top traces), in contrast to ADP binding that resulted in an exothermic (negative) signal (Figure 2A, bottom traces). Figure 2B and Table 1 show the binding isotherms and the thermodynamic parameters obtained at 25 °C using a single-site binding model. The apparent Gibbs free energy (ΔG_{app}) for ATP and ADP binding is similar, the affinity for ATP being slightly higher. The unfavorable enthalpy change (ΔH) for ATP binding is compensated by an unusually large entropic term ($-T\Delta S$). It should be noted that the binding enthalpy was similar regardless of the ionization enthalpy of the buffer used (not shown), indicating that no protonation effects were linked to the binding reaction.

It has been reported that inorganic phosphate (P_i) reduces the rate of ADP dissociation (*23*−*25*). Titration experiments in the presence of low (2 mM) and high (10 mM) P_i concentrations were conducted to investigate the effect of P_i on ADP binding thermodynamics. While 2 mM P_i had little effect on ADP binding, higher concentrations (10 mM) increased 2-fold the binding enthalpy change and decreased the affinity for ADP

Table 1: Thermodynamic Parameters of Nucleotide Binding to DnaK, $DnaK_{T199A}$, $DnaK(1-507)_{T199A}$, and $K\text{-}NBD_{T199A}$ at 25 °C[a]

	NTP	K_d (nM)	ΔG_{app} (kcal/mol)	ΔH_{app} (kcal/mol)	$-T\Delta S_{app}$ (kcal/mol)	ΔC_p[b] (kcal K^{-1} mol^{-1})
wt DnaK	AMP-PNP	925	−8.2	−1.3	−6.9	−
	ATPγS	280	−9.0	−2.4	−6.6	−
$DnaK_{T199A}$	ATP	160	−9.3	9.0	−18.3	−
	ADP	250	−9.0	−3.2	−5.8	−
	ADP with 2 mM P_i	240	−9.0	−3.7	−5.3	−
	ADP with 10 mM P_i	740	−8.4	−7.3	−1.0	−
$K\text{-}NBD_{T199A}$	ATP	610	−8.9	−4.1	−3.7	0.07
	ADP	170	−9.2	−3.3	−6.0	−0.05
$DnaK(1-507)_{T199A}$	ATP	92	−9.6	17.0	−26.6	−
	ADP	230	−9.0	−3.2	−5.8	−

[a]Experiments were repeated at least twice. The maximum standard deviation in the resulting parameter was 10−20% for K_d and 0.3 kcal/mol for ΔH and $-T\Delta S$. [b]ΔC_p values for $DnaK_{T199A}$ and $DnaK(1-507)_{T199A}$ are not given because of the nonlinear dependence of ΔH on temperature.

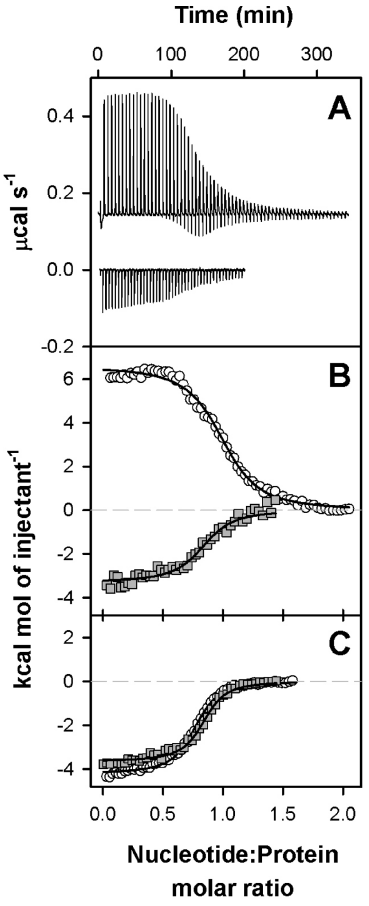

FIGURE 2: ATP and ADP titration of $DnaK_{T199A}$ and $K\text{-}NBD_{T199A}$. (A) Baseline-corrected instrumental response of successive injections of ATP (top trace) and ADP (bottom trace) into a solution of $DnaK_{T199A}$ at 25 °C. Protein and nucleotide concentrations were as indicated in Materials and Methods. Buffer was as in Figure 1. ATP titration was shifted 0.1 μcal/s for better viewing. (B) Integrated data of $DnaK_{T199A}$ titration with ATP (circles) and ADP (squares) and fit of the corresponding binding isotherms to a single-site binding model (solid lines). (C) Same as panel B for $K\text{-}NBD_{T199A}$.

(Table 1). These effects can be explained assuming a K_d for P_i in the low millimolar range (*23*, *24*) and that partial occupation of the nucleotide binding site by P_i reduces the affinity for ADP.

We next assessed nucleotide binding to $K\text{-}NBD_{T199A}$. In contrast to what was found for the full-length protein, the isotherms of ATP and ADP binding to the NBD were both exothermic (Figure 2C), with only a small difference in the

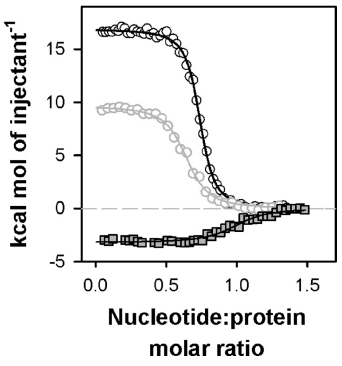

FIGURE 3: Titration of deletion construct $DnaK(1-507)_{T199A}$. Isotherms for ATP (empty black circles) and ADP (gray squares) binding to $DnaK(1-507)_{T199A}$ and fit of the experimental data to a single-site binding model (solid lines). For the sake of comparison, the ATP binding isotherm for $DnaK_{T199A}$ is shown (empty gray circles).

contribution of the enthalpic and entropic terms to the free energy of binding (Table 1). Interestingly, the thermodynamic parameters obtained from these isotherms are very similar to those estimated for ADP binding to DnaK (Table 1).

In summary, the data described above provide evidence that ATP-induced interdomain allosteric communication contributes to the experimental enthalpy and entropy changes in full-length DnaK. The effect of ADP seems to be limited to the NBD since no significant differences are observed when nucleotide binding to the full-length protein and to the isolated NBD is compared.

Effect of the Lid Subdomain on ATP Binding Thermodynamics. Several studies have proposed the involvement of the lid subdomain of DnaK SBD in the functionally essential, nucleotide-induced allosteric conformational changes (*12*, *14*, *16*). To estimate the contribution of this subdomain to the thermodynamics of nucleotide binding, we performed titration experiments with a ΔLid construct containing the T199A mutation [$DnaK(1-507)_{T199A}$]. As observed with the full-length protein, ATP binding to the deletion mutant was an endothermic reaction that, however, exhibited a significantly larger enthalpy change (Figure 3 and Table 1). In contrast, ADP binding to $DnaK(1-507)_{T199A}$ yielded binding isotherms and thermodynamic parameters similar to those estimated for the full-length protein and the isolated NBD domain. These results suggest that the ATP-induced interdomain interactions differ in the ΔLid construct and the full-length protein.

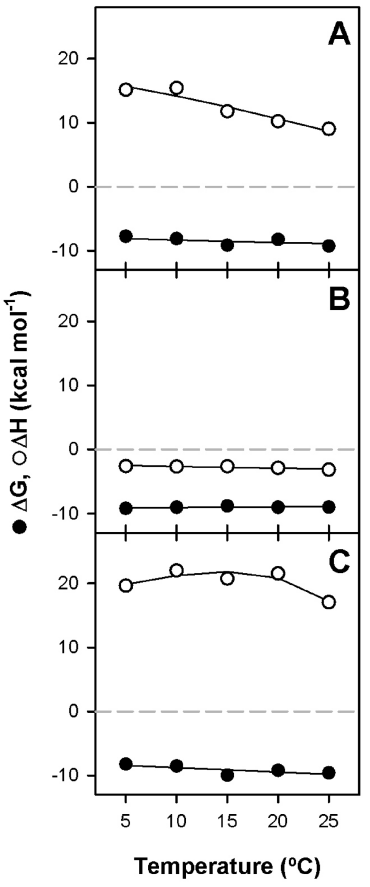

FIGURE 4: Temperature dependence of the apparent binding enthalpy and Gibbs free energy. Experimental values of ΔH_{app} and ΔG_{app} at different temperatures for ATP (A) and ADP (B) binding to $DnaK_{T199A}$ and ATP (C) binding to $DnaK(1-507)_{T199A}$. The temperature dependence of the enthalpy and Gibbs free energy is fitted (solid lines) to the nucleotide binding model described in the Appendix.

Temperature Dependence of Nucleotide Binding and Binding Model. The temperature dependence of the thermodynamic parameters of nucleotide binding to $DnaK_{T199A}$, K-NBD_{T199A}, and $DnaK(1-507)_{T199A}$ has also been investigated (Figure 4). While ΔH and ΔG of ATP binding to the full-length protein showed a strong temperature dependence, ADP binding did not over the temperature range studied (Figure 4A,B). A curvature of the plots of the apparent ΔH and ΔG with temperature was observed for ATP binding to the ΔLid construct (Figure 4C). Deviation from linearity indicates the existence of a nucleotide-induced, temperature-dependent conformational change coupled to the binding process (*26*). The apparent ΔH of ATP and ADP binding to K-NBD_{T199A} did not vary with temperature, resulting in small values for the heat capacity change, ΔC_p (Table 1).

To estimate the contribution of the nucleotide-induced conformational change to the apparent binding thermodynamic parameters, a two-state model for the interaction with each nucleotide has been considered, as described in the Appendix. In the absence of nucleotides, apoDnaK can populate three different conformational states in equilibrium: (i) an initial reference state ($DnaK^0$), (ii) an open conformation ($DnaK^T$) that interacts with peptide substrates with low affinity and fast binding and release kinetics, and (iii) a closed state ($DnaK^R$) with high affinity for peptide substrates and slow exchange kinetics. ATP or ADP binding would stabilize $DnaK^T$ or $DnaK^R$, respectively, shifting the equilibrium toward the corresponding nucleotide-bound conformation:

$$DnaK^T(ATP) \xleftarrow{+ATP} DnaK^T \leftrightarrow DnaK^0 \leftrightarrow DnaK^R \xrightarrow{+ADP} DnaK^R(ADP)$$

Thus, there is a conformational equilibrium coupled to nucleotide binding. Each of these two processes will be characterized by their corresponding thermodynamic parameters: association or equilibrium constant, Gibbs energy, enthalpy, entropy, and heat capacity changes. The apparent experimental thermodynamic parameters for nucleotide binding will thus be modulated by the conformational equilibrium.

Assuming the thermodynamic parameters for nucleotide binding to K-NBD as a reasonable approximation of the intrinsic binding parameters in the absence of conformational change (Table 1) and fitting the experimental data obtained at different temperatures (Figure 4) to eqs 10 and 11 (Appendix), we can estimate the thermodynamic parameters for the conformational equilibrium coupled to nucleotide binding (Table 2). In the absence of nucleotide, <20% of the protein ($K^T_{conf} = 4.4$) would be in the open state ($DnaK^T$), whereas almost 90% ($K^R_{conf} = 0.1$) would be in the closed state ($DnaK^R$) at 25 °C. Therefore, from an energetic point of view, $DnaK^0$ is similar to $DnaK^R$, as also suggested by different techniques (*14*, *20*, *21*). An estimation of K_{conf} for the T ↔ R conformational transition can be obtained from the corresponding K_{conf} values for each equilibrium (see the Appendix):

$$K^{T\leftrightarrow R}_{conf} = \frac{K^T_{conf}}{K^R_{conf}} = 44$$

These results indicate that the R conformation is the most populated because of its lower conformational free energy. The T conformation of the ΔLid construct is also energetically distinguishable from the other states, only one-third of the deletion mutant ($K^T_{conf} = 2.0$) being in the T state at 25 °C.

The solvation and conformational contributions to the entropic term can be dissected using the value of $\Delta C_p{}^{conf}$ and eqs 12 and 13 in the Appendix. The solvation term reflects the gain or loss of degrees of freedom of water molecules due to (de)solvation of molecular binding interfaces. The conformational entropy reflects the molecular degrees of freedom of the protein [e.g., partial (un)folding, immobilization of rotatable bonds, etc.]. The estimated changes in the solvation and conformational entropies (Table 2) indicate that (i) the T → 0 conformational transition is accompanied by solvation, i.e., incorporation of water molecules on the protein surface, and disordering of the molecule, (ii) the R → 0 conformational transition is characterized by desolvation and partial ordering, and (iii) deletion of the lid induces a change in the sign of the entropic term for the T → 0 transition, which proceeds with desolvation of the protein surface and ordering of the molecule.

DISCUSSION

To perform their function, Hsp70 proteins cycle between two main conformational states: an ATP-bound state (T), with low affinity for peptide substrates and fast exchange kinetics, and an ADP-bound conformation (R), with high affinity and slow binding and release kinetics. The actual view of protein dynamics suggests that a protein can access multiple conformations, with a probability that will depend on the energetic content of each conformation. Therefore, DnaK in the absence of nucleotides could sample, among others, the T and R conformations. These different states are in equilibrium, their relative abundance

Table 2: Thermodynamic Parameters of the DnaK and DnaK(1−507) Conformational Equilibrium Coupled to Nucleotide Binding at 25 °C

						$-T\Delta S$ (kcal/mol)	
		K_{conf}	$\langle\Delta G_{conf}\rangle^a$ (kcal/mol)	ΔH_{conf} (kcal/mol)	ΔC_p^{conf} (kcal K^{-1} mol^{-1})	$-T\Delta S_{sol}$	$-T\Delta S_{conf}$
DnaK	$K^T \leftrightarrow K^0$	4.4	−1.0	−14.0	0.29	13.1	
						22	−9
	$K^R \leftrightarrow K^0$	0.1	−0.06	−2.1	−0.19	3.3	
						−14.5	17.8
DnaK(1−507)	$K^T \leftrightarrow K^0$	2.0	−0.65	−32.0	−0.31	31.6	
						−24	55

[a] $\langle\Delta G_{conf}\rangle$ represents the conformational penalty to the binding process and is calculated from $-RT\ln(1 + K_{conf})$ (see the Appendix). It should be noted that it is different from ΔG_{conf} which equals $-RT\ln(K_{conf})$.

depending on their conformational free energy. The existence of a conformational equilibrium between different DnaK states has been previously proposed to explain the ATP binding kinetics monitored by DnaK intrinsic fluorescence quenching (*24*, *27*). ATP and ADP binding quantitatively shift the conformational equilibrium toward the T and R states, respectively.

We find that the R state is largely populated in the absence of ADP due to its low conformational free energy (Table 2). This finding agrees with previous studies that showed small differences between the free and ADP-bound protein (*14*, *20*, *21*). In contrast, the conformational transition to the T state is energetically unfavorable, and therefore, this conformation is sparsely populated. ATP binding compensates for the conformational free energy penalty (approximately 1 kcal/mol), making the T state energetically accessible. This value is in the range of the estimated energy provided by ligand binding to proteins, which usually is a few kilocalories per mole (*28*). As expected, the difference in conformational stability between the T and R states is not large, since (i) both conformations must be accessible during the functional cycle of the protein and, thus, cannot be separated either thermodynamically by a large energy difference or kinetically by a high energy barrier and (ii) the affinity and apparent binding free energy (ΔG) are similar for both nucleotides. Reported K_d values for ATP are highly variable (*21*−*23*, *29*). Our estimation of the ATP dissociation equilibrium constant (0.16 μM), measured by direct titration of $DnaK_{T199A}$ with ATP, is in agreement with that obtained by internal chemical cross-linking of DnaK in the presence of ATP (0.19 μM) (*21*). In contrast, the K_d estimated by measurement of k_{on} and k_{off} rate constants resulted in much lower values [1 nM (*23*) and 7 nM (*22*)]. Interestingly, Theyssen and co-workers found a K_d upper limit of 0.22 μM in the same work, when ATP hydrolysis was not taken into account (*22*), which agrees with the K_d described here for the ATPase deficient mutant $DnaK_{T199A}$. It is also likely that the affinity of $DnaK_{T199A}$ for ATP is slightly lower compared to that of the wild-type protein since T199 is involved in the binding of the γ-phosphate group in hsc70 (*30*).

The contribution of the enthalpic (ΔH) and entropic terms ($-T\Delta S$) to the ADP and ATP binding free energy is different. ADP binding is an exothermic process, favored by changes in both enthalpy and entropy. The fact that ADP binding to the wt protein and deletion mutants DnaK(1−507) and K-NBD shows similar thermodynamic parameters is in accordance with a nucleotide-induced conformational change limited to the NBD (*11*, *20*). The effect of the SBD on ADP binding to the NBD is expected to be minimal since both domains behave independently in the R state (*17*, *18*). The source of the favorable enthalpic contribution to ADP binding to the NBD might be multiple bonding interactions between the nucleotide and the protein, as observed in the three-dimensional structure of the ADP-bound Hsc70 homologue (*30*, *31*), and the formation of two salt bridges between subdomains IB and IIB that close the nucleotide binding cleft (*32*). The increase in the ADP binding enthalpy observed in the presence of 10 mM P_i might reflect a larger number of electrostatic interactions and/or a slightly different conformation of the NBD as suggested by DnaK intrinsic fluorescence (*22*). In contrast, ATP binding proceeds with an unfavorable enthalpy change and is driven by the entropic term. The lack of a high-resolution structure of the ATP-bound conformation hampers an explanation of this behavior. The entropic term provides information about the gain and loss of degrees of freedom of solvent molecules bound to the protein surface and protein conformational changes, when decomposed into solvation and conformational terms, respectively (loss of roto-translational entropy is not considered in a conformational equilibrium). Interestingly, for the $DnaK^T \leftrightarrow DnaK^0$ conformational equilibrium, we find that $\Delta S_{sol} < 0$, supporting solvation of protein areas that were previously interacting, and $\Delta S_{conf} > 0$, indicating an overall disordering of DnaK. These results are compatible with recent reports showing that ATP binding induces docking of the SBD and the hydrophobic linker onto the NBD (*16*, *17*). Since in the ADP conformation (R) both domains are separated and connected by a flexible linker (*18*), the conformational change from T to R (or 0, which is very similar to R) should result in solvation and disordering of the interacting surfaces, as we observe. Gierash and co-workers (*17*) also found that ATP binding partially disorders residues of the substrate binding site in the SBD. However, our results suggest that this effect should be less pronounced that the global ordering induced by domain docking.

A comparison of the data obtained with the ΔLid construct and the full-length protein reveals the following important differences. First, the magnitude of the endothermic signal associated with ATP binding to ΔLid is larger. Although this construct still maintains an ATP-induced allosteric conformational change (*14*, *19*), the higher binding enthalpy indicates a different nucleotide-induced intramolecular rearrangement. Second, the sign of ΔS_{sol} and ΔS_{conf} for the $T \rightarrow 0$ conformational transition is opposite, meaning that upon ATP binding to the ΔLid construct solvation does not occur and the molecule becomes less ordered. These results suggest that lid removal diminishes or abolishes domain docking in the T protein state. Helices αA and αB of the lid subdomain are very likely to participate in the interdomain interface as evidenced by changes in the DnaK intrinsic fluorescence and deuteron incorporation experiments (*14*, *16*). Moreover, the structure of the ADP−substrate−DnaK complex, recently published by Zuiderweg and co-workers (*18*), maps helix αA in the proximity of subdomain IA of the NBD. These results support the participation of the lid subdomain in interdomain interactions in the T state. We also

find that, contrary to what is observed for the full-length protein, the deletion mutant is more disordered in the T state ($\Delta S_{conf} < 0$), suggesting that lid deletion increases the conformational heterogeneity of the ATP state. It has also been described that in the absence of the lid subdomain there is no interdomain communication at elevated temperatures, so that ATP binding to the ΔLid construct does not induce a conformational change in the SBD (*20*). In this context, the values of K_{conf} at 37 °C for the T → 0 transition can be obtained from the values at 25 °C and eq 10 of the Appendix. While the energetic difference between these states is still significant for the full-length protein [K_{conf}(37 °C) = 2.0; only 30% of the protein would adopt the T conformation], the lack of interdomain communication in the ΔLid construct greatly diminishes the energy penalty [K_{conf}(37 °C) = 0.2; 83% of the protein would be in the T state], as found for the R state of the wt protein [K_{conf}(37 °C) = 0.1]. Therefore, the T and R states of DnaK(1−507) are conformationally similar at elevated temperatures (i.e., 37 °C), and thus, the conformational change coupled to nucleotide binding is abolished.

The (un)foldase activity (*3*, *33*) of Hsp70 proteins involves remodeling of non-native interactions that occur in protein folding intermediates and aggregates, an event that requires energy. We find in this work that the open state of $DnaK^T$ is energetically unfavorable and ATP binding surmounts an energy penalty ($\langle\Delta G_{conf}\rangle$) of approximately 1 kcal/mol that is stored upon ATP binding. The Gibbs free energy for the T → R transition estimated from $K_{conf}^{T\leftrightarrow R}$ ($\Delta G = -RT \ln K_{conf}^{T\leftrightarrow R}$) would be −2.2 kcal/mol at 25 °C, an energy that could be used to partially remodel the substrate, in addition to the free energy of ATP hydrolysis (approximately −7 kcal/mol). This value agrees with that estimated by Christen and co-workers (*34*) from the affinity difference between the T and R states for a short peptide (−2 kcal/mol). The magnitude of this conformational free energy might not be enough to remodel a non-native protein conformation (protein unfolding usually requires 5−10 kcal/mol), but it is significant if we consider that more than one DnaK molecule can act on one substrate protein and that only local regions of the substrate are rearranged at a time.

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APPENDIX

Nucleotide Binding Model. We consider a two-state model for nucleotide binding to DnaK, where we define a conformational equilibrium between an initial state unable to bind nucleotide ($DnaK^0$) and an open ($DnaK^T$, ATP binding competent) or closed ($DnaK^R$, ADP binding competent) conformation of DnaK. ATP or ADP binding to DnaK shifts the equilibrium quantitatively toward the T or R state, respectively:

$$DnaK^0 \leftrightarrow DnaK^T \xrightarrow{+ATP} DnaK^T(ATP)$$

$$DnaK^0 \leftrightarrow DnaK^R \xrightarrow{+ADP} DnaK^R(ADP)$$

Therefore, there is a conformational equilibrium coupled to a binding process. Each process will be characterized by its corresponding thermodynamic parameters: association constant or equilibrium constant, Gibbs energy, enthalpy, entropy, and heat capacity changes. The apparent thermodynamic parameters for nucleotide binding will be modulated by the conformational equilibrium. For convenience, we represent the initial state ($DnaK^0$) as A and the open ($DnaK^T$) or closed state ($DnaK^R$) as B, which is considered the reference state. The equilibrium between both conformational states is governed by the conformational equilibrium constant, K_{conf}, defined by

$$B \overset{K_{conf}}{\leftrightarrow} A \quad K_{conf}(T) = \frac{[A]}{[B]} = \frac{F_A}{F_B} \tag{1}$$

where F_A and F_B are the fractions of states A and B, respectively (concentration of A or B over the total protein concentration), and (T) represents the temperature dependence of the equilibrium constant. These fractions can be expressed in terms of the equilibrium constant:

$$F_A = \frac{K_{conf}(T)}{1+K_{conf}(T)} \quad F_B = \frac{1}{1+K_{conf}(T)} \tag{2}$$

Nucleotide (N) binding to the B state of DnaK (ATP to $DnaK^T$ state and ADP to $DnaK^R$) is governed by the intrinsic association constant, $K_N^0(T)$, given by

$$B \overset{+N}{\leftrightarrow} BN \quad K_N^0(T) = \frac{[BN]}{[B][N]} \tag{3}$$

The mass conservation equations for protein and nucleotide are

$$\begin{aligned}[DnaK]_T &= [A]+[B]+[BN]\\ [N]_T &= [N]+[BN]\end{aligned} \tag{4}$$

via introduction of the chemical equilibrium equations (eqs 1 and 3) in the previous equation, the total concentration of nucleotide (ATP or ADP) is given by

$$\begin{aligned}[N]_T &= [N]+[DnaK]_T\frac{K_N^0(T)[N]}{1+K_{conf}(T)+K_N^0(T)[N]}\\ &= [N]+[DnaK]_T\frac{K_N^{app}(T)[N]}{1+K_N^{app}(T)[N]}\end{aligned} \tag{5}$$

where the second term in the right-hand part of this equation represents the concentration of bound nucleotide. The apparent association constant for nucleotide binding in the presence of a conformational change is defined as

$$K_N^{app}(T) = \frac{K_N^0(T)}{1+K_{conf}(T)} \tag{6}$$

As expected, this equation indicates that the binding affinity decreases if the conformational equilibrium constant increases, that is, if the population of state A ($DnaK^0$, unable to bind nucleotide) increases. The apparent Gibbs energy of binding is given by

$$\begin{aligned}\Delta G_N^{app}(T) &= -RT \ln K_N^{app}(T) = \Delta G_N^0(T)+RT\ln[1+K_{conf}(T)]\\ &= \Delta G_N^0(T)-\langle\Delta G_{conf}(T)\rangle\end{aligned} \tag{7}$$

where ΔG_N^0 is the intrinsic Gibbs energy of binding in the absence of a conformational equilibrium. The second term in the right-hand part of the equation, $\langle\Delta G_{conf}\rangle$, is the average excess conformational Gibbs energy and is given by $-RT$ ln[1 + $K_{conf}(T)$]. Considering B as the reference state, if K_{conf} is greater

than 1 (state A is more populated than state B), then the conformational Gibbs energy represents a penalty to the overall free energy of binding. The apparent enthalpy of binding is obtained applying the Gibbs−Helmholtz relationship:

$$\Delta H_{\mathrm{N}}^{\mathrm{app}}(T) = -T^2\frac{\partial}{\partial T}\left[\frac{\Delta G_{\mathrm{N}}^{\mathrm{app}}(T)}{T}\right] = \Delta H_{\mathrm{N}}^{0}(T) - \frac{K_{\mathrm{conf}}(T)}{1+K_{\mathrm{conf}}(T)}\Delta H_{\mathrm{conf}}(T) \tag{8}$$

$$= \Delta H_{\mathrm{N}}^{0}(T) - F_{\mathrm{A}}(T)\Delta H_{\mathrm{conf}}(T) = \Delta H_{\mathrm{N}}^{0}(T) - \langle \Delta H_{\mathrm{conf}}(T)\rangle$$

where $\Delta H_{\mathrm{N}}^{0}$ is the intrinsic binding enthalpy in the absence of a conformational equilibrium and ΔH_{conf} is the conformational enthalpy. The apparent binding heat capacity change is given by the temperature derivative of the apparent binding enthalpy:

$$\Delta C_{p,\mathrm{N}}^{\mathrm{app}}(T) = \frac{\partial}{\partial T}[\Delta H_{\mathrm{N}}^{\mathrm{app}}(T)] \tag{9}$$

$$= \Delta C_{p,\mathrm{N}}^{0}(T) - \frac{K_{\mathrm{conf}}(T)}{1+K_{\mathrm{conf}}(T)}\Delta C_{p,\,\mathrm{conf}}(T) - \frac{K_{\mathrm{conf}}(T)}{[1+K_{\mathrm{conf}}(T)]^2}\frac{\Delta H_{\mathrm{conf}}(T)^2}{RT^2}$$

$$= \Delta C_{p,\mathrm{N}}^{0}(T) - F_{\mathrm{A}}(T)\Delta C_{p,\,\mathrm{conf}}(T) - F_{\mathrm{A}}(T)F_{\mathrm{B}}(T)\frac{\Delta H_{\mathrm{conf}}(T)^2}{RT^2}$$

$$= \Delta C_{p,\mathrm{N}}^{0}(T) - \langle \Delta C_{p,\,\mathrm{conf}}(T)\rangle$$

where $\Delta C_{p,\mathrm{N}}^{0}$ is the intrinsic binding heat capacity in the absence of a conformational equilibrium and $\Delta C_{p,\mathrm{conf}}$ is the conformational heat capacity.

Equations 7−9 indicate that the experimental apparent binding affinity, enthalpy, and heat capacity will deviate from the intrinsic binding parameters if there is a significant population of state A. The temperature dependence of the conformational transition will be given by the following expressions:

$$K_{\mathrm{conf}}(T) = K_{\mathrm{conf}}(T_0)\exp\left\{-\frac{\Delta H_{\mathrm{conf}}(T_0)}{RT}\left(1-\frac{T}{T_0}\right) - \frac{\Delta C_{p,\,\mathrm{conf}}}{R}\left[1-\frac{T_0}{T}-\ln\left(\frac{T}{T_0}\right)\right]\right\}\Delta G_{\mathrm{conf}}(T)$$

$$= -RT\ln K_{\mathrm{conf}}(T)\Delta H_{\mathrm{conf}}(T)$$

$$= \Delta H_{\mathrm{conf}}(T_0) + \Delta C_{p,\,\mathrm{conf}}(T-T_0) \tag{10}$$

and that of the binding equilibrium by

$$K_{\mathrm{N}}^{0}(T) = K_{\mathrm{N}}^{0}(T_0)\exp\left\{-\frac{\Delta H_{\mathrm{N}}^{0}(T_0)}{RT}\left(1-\frac{T}{T_0}\right) - \frac{\Delta C_{p,\mathrm{N}}^{0}}{R}\left[1-\frac{T_0}{T}-\ln\left(\frac{T}{T_0}\right)\right]\right\}\Delta G_{\mathrm{N}}^{0}(T)$$

$$= -RT\ln K_{\mathrm{N}}^{0}(T)\Delta H_{\mathrm{N}}^{0}(T)$$

$$= \Delta H_{\mathrm{N}}^{0}(T_0) + \Delta C_{p,\mathrm{N}}^{0}(T-T_0) \tag{11}$$

where T_0 is a reference temperature (e.g. 298.15 K). It has been assumed that the intrinsic binding and conformational heat capacities are constant (at least over the experimental temperature range considered).

From the heat capacity change between states A and B, we can estimate the solvation entropy associated with the conformational change coupled to nucleotide binding:

$$-T\Delta S_{\mathrm{sol}} = -T\Delta C_{p,\,\mathrm{conf}}\ln\left(\frac{T}{385.15}\right) \tag{12}$$

The total entropy change for the conformational equilibrium between states A and B can be partitioned into solvation and conformational terms (assuming that the roto-translational entropy does not apply to the conformational change and no other coupled equilibria are present):

$$-T\Delta S = -T\Delta S_{\mathrm{sol}} - T\Delta S_{\mathrm{conf}} \tag{13}$$

in which the solvation entropy reflects the gain or loss of degrees of freedom of solvent molecules due to (de)solvation of the molecular binding interfaces and the conformational entropy reflects the gain or loss of internal degrees of freedom of the protein−nucleotide complex. Therefore, the conformational entropy associated with the conformational change coupled to nucleotide binding may be estimated from eq 14:

$$-T\Delta S_{\mathrm{conf}} = -T\Delta S - (-T\Delta S_{\mathrm{sol}}) \tag{14}$$

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