NMR study of nucleotide-induced changes in the nucleotide binding domain of *Thermus Thermophilus* Hsp70 chaperone DnaK: implications for the allostERIC MECHANISM

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Running Title: Nucleotide-induced Structural Changes in DnaK

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Abstract

We present an NMR investigation of the nucleotide dependent conformational properties of a 44 kDa nucleotide binding domain (NBD) of an Hsp70 protein. Conformational changes driven by ATP binding and hydrolysis in the N-terminal NBD are believed to allosterically regulate substrate affinity in the C-terminal substrate binding domain. Several crystal structures of Hsc70 NBD's in different nucleotide states have, however, not shown significant structural differences. We have previously reported the NMR assignments of the backbone resonances of the NBD of the bacterial Hsp 70 homologue *Thermus thermophilus* DnaK in the ADP bound state. In this study we show, by assigning the NBD with the ATP/transition state analogue, ADP-AlF₄⁻, bound, that it closely mimics the ATP-bound state. Chemical shift difference mapping of the two nucleotide states identified differences in a cluster of residues at the interface between subdomains 1A and 1B. Further analysis of the spectra revealed that the ATP state exhibited a single conformation while the ADP state was in slow conformational exchange between a form similar to the ATP state and another state unique to the ADP bound form. A model is proposed of the allosteric mechanism based on the nucleotid state altering the balance of a dynamic equilibrium between the open and closed states. The observed chemical shift perturbations were concentrated in an area close to a previously described J-domain binding channel, confirming the importance of that region in the allosteric mechanism.

The abbreviations used are: Hsp70, 70 kDa heat shock protein; TTH, *Thermus thermophilus*; TTK, *Thermus thermophilus* DnaK; NBD, nucleotide binding domain; SBD, substrate binding domain; NMR, nuclear magnetic resonance spectroscopy; TROSY, transverse relaxation-optimized spectroscopy
Introduction

The Hsp70 (70 kDa heat shock proteins) family of protein chaperones are involved in a wide variety of crucial cellular functions that involve protein folding and unfolding. Due to the diversity of possible interactions the activity and specificity of these proteins are subject to regulation on several levels. Under normal growth conditions these proteins play a critical role in many cellular processes such as translocation across membranes and binding to nascent polypeptide chains to promote correct folding (1). Under cell stress conditions such as heat shock the Hsp70 proteins are highly expressed and are highly active in protein rescue and triage processes that are vital to cell survival. Most organisms express multiple versions of these proteins; some are highly expressed in response to cell stress conditions such as *Escherichia coli* DnaK and mammalian Hsp70 (2) while other homologues carry out specific constitutive functions in the cytoplasm, such as Hsc70 (2), or in the endoplasmic reticulum, BiP (3) for example. Multiple cochaperones regulate Hsc70 activity through substrate binding and nucleotide hydrolysis (the DnaJ/Hsp40 family (4,5)) and nucleotide exchange (GrpE and Bag domain proteins (6,7)). In the absence of co-chaperones the activity of all known Hsp70 proteins is regulated through an intramolecular allosteric mechanism where the bound nucleotide state (ADP or ATP) at one site on the molecule determines the affinity for protein substrate at another location (8). Biochemical and structural studies of the Hsp70 family have led to insights about the interactions with nucleotide exchange factors and the chaperone activity but have not provided substantial structural details of the intramolecular allosteric regulation(9).

Hsp70 proteins consist of two major structural elements, an N-terminal 44 kDa nucleotide binding domain (NBD) connected by a conserved, hydrophobic 10 - 12 amino acid linker to a 26 kDa substrate binding domain (SBD). Crystal structures of the NBD (10) revealed
that it has an overall V-shape with a subdomain of about equal size on either side of a deep nucleotide binding cleft (Fig. 1). The SBD consists of a 13 kDa β-sandwich, which contains the hydrophobic substrate binding site, followed by a 12 kDa C-terminal domain that forms a helical lid over the substrate binding pocket. The structure of the SBD has been characterized by both NMR spectroscopy and X-ray crystallography (11-14) and in both apo and substrate bound states. The substrate binding site exhibits an affinity for hydrophobic protein sequences that would not be solvent exposed in native proteins (15). The chaperone activity of the Hsc70 class of protein depends on the binding of these sequences by the SBD to interfere with the tendency of these exposed regions to cause the formation of non-native conformations or aggregates (16). No detailed structural information for a complete Hsp70 protein is available, therefore, the sites and nature of the interdomain interactions are still unknown.

DnaK from the thermophilic eubacterium *Thermus Thermophilus* (TTK) is believed to be a structural and functional homologue to the well-characterized *E. coli* DnaK and bovine Hsc70 proteins(17). Functional assays have demonstrated that TTK refolds denatured proteins in a manner similar to other Hsc70 proteins (18,19) and that it interacts with a similar set of co-chaperones (20) that modulate its activity. TTK is structurally stable up to 95 °C and is a functional chaperone over a temperature range from 25 to at least 75 °C (18). Unlike the *E. coli* DnaK and bovine Hsc70, however, TTK exists in vivo in a stable 3:3:3 complex with TTH DnaJ and with DafA, a co-factor that is unique to *T. Thermophilus* (21). More recent work suggests that both monomeric and complexed forms are present in vivo and that both forms are functional chaperones but differ in their relative abilities to disaggregate denatured proteins(22).

This report compares backbone NMR assignments of the 44 kDa nucleotide binding domain of TTK (23) in the two nucleotide states to identify the residues exhibiting
conformational differences. By using modern TROSY-based triple resonance experiments at 800 MHz NMR spectrometer frequencies, we have been able to obtain more than 90% of the backbone assignments ($^{1}H\alpha$, $^{15}N\alpha$, $^{13}C\alpha$, $^{13}CO$, $^{13}C\beta$) of the NBD of TTK at 55 °C in both the ADP-Pi state (23) and, in this report, complexed with ADP-AlF$_4$ (an ATP or ATP/ADP transition state mimic). While TTK has not been structurally characterized it does have a high level of sequence homology (20) to the *E. coli* DnaK and bovine Hsc70 NBD’s that have atomic resolution structures available. In addition, chemical shift-based prediction of secondary structure matched that observed in the crystal structure of the Hsc70 NBD (23). Based on this information, a reliable homology model of TTK NBD could be constructed based on the crystal structure of Hsc70 NBD, which is presented herein.

Comparison of the backbone NMR chemical shifts of NBD with ADP-Pi or ATP bound indicated a high degree of similarity with only 15 resonances changing significantly, suggesting that there was limited structural differences between the two states. A more interesting observation was that in the ADP state more than 50 residues produced broadened or doubled peaks, suggesting a slow dynamic equilibrium between two conformations with $\Delta G \sim 0$, while in the ATP state only one conformation was indicated for these residues. Detailed characterization of the ATP state required the use of ADP-AlF$_4$ as a non hydrolyzable ATP analogue that produced spectra almost identical to that of the ATP-bound TTK. The availability of resonance assignments and a reliable homology model allowed us to map the altered resonances between the different nucleotide states on the protein structure, leading to novel insights into the dynamic nature of the allosteric mechanism.
Experimental Procedures

Cloning of His-Tagged T. Thermophilus DnaK Nucleotide-binding Domain-The protein used in this study consisted of the TTK 1-381 sequence with the addition of a 20 amino acid N-terminal leader sequence that included a 6 residue histidine tag and a thrombin cleavage site. The plasmid, pMR20, was constructed as described previously (23).

Expression and Purification of T. thermophilus DnaK Nucleotide-binding Domain-E. coli strain BL21(DE3) cells (Novagen, Madison, WI) containing the pMR20 plasmid were grown and expressed as described in Revington and Zuiderweg(23). NMR samples used for assignment purposes were $^{2}$H, $^{15}$N, $^{13}$C labeled and were denatured/refolded during purification to allow protonation of buried amide residues. The denaturation/refolding process also allowed complete removal of the nucleotide. TROSY spectra of non-denatured samples were compared to those of the refolded protein to ensure that it had regained the native conformation. Samples used for titrations and proteolytic testing were $^{15}$N labeled. The NMR samples were in 50 mM HEPES buffer pH 7.4, 10 mM KCl and 5 mM MgCl$_2$.

Final concentration of the assignment samples was 450 to 600 µM while the $^{15}$N samples used for other experiments were between 100-1500 µM. For the assignment samples, the nucleotide to protein molar ratios were approximately 10:1. In the ADP state a molar equivalent of inorganic phosphate to ADP was also added producing the more stable ADP-Pi state. Protein concentration was measured based on a theoretical extinction coefficient at 280 of 11,200 M$^{-1}$ cm$^{-1}$ (24). The concentration of ATP and ADP used in the nucleotide titrations was estimated by $E_{259}$ of 15,300 M$^{-1}$cm$^{-1}$ (25).

ADP or ATP was introduced to samples of TTK-NBD in the apo form by adding small aliquots of a 100 mM stock solution in the same buffer and pH. AlF$_3$ was introduced in the
sample by preparing a dilute solution (2-5 µM) of apo NBD in a total volume of 50 ml. AlCl₃ and ADP were added to a final concentration of 200 µM and NaF was added to 10 mM. The solution was stirred for 30 minutes at room temperature and then concentrated by ultrafiltration to 0.5 ml for NMR studies.

**NMR Experiments** - NMR spectra, except where otherwise noted, were collected at 55 °C on an 800 MHz Varian Inova spectrometer, using a regular triple-resonance gradient probe, and using samples of about 400 µM in protein. For the assignments of the ADP bound form of TTK in-house versions of the standard set of three dimensional, triple resonance pulse sequences with TROSY detection and ²H decoupling were used for all experiments (23). Using the ADP backbone assignments as a starting point, the ADP-AlFx form was assigned based on TROSY-HNCA, TROSY-HNCACB, TROSY-HNCO and TROSY-HNCA CO. All spectra were initially processed with NMRPipe (26) and analyzed using XEASY(27), Felix (Accelrys, San Diego, CA) or Sparky (28).

**Nucleotide titrations and chemical shift mapping** - We have completed the backbone resonance assignments for the ADP (23) and ADP-AlFx forms of TTK-NBD but not the apo state. Quantitative ADP and ATP titrations were carried out by adding small aliquots of concentrated nucleotide solutions to a solution of 900 µM apo form of TTK-NBD and collecting 2D ¹H-¹⁵N TROSY spectra with protein:nucleotide ratios of 3:0; 3:1, 3:2, 3:3, 3:6 and 3:15.

The chemical shift mapping of the changes in the ¹H-¹⁵N TROSY spectra between the ADP and ADP-AlFx states was accomplished by calculating the combined chemical shift difference, Δδ(NH), between assigned resonances in the two forms with equation 1 (29):

$$\Delta \delta(NH) = [(\Delta \delta H^2 + (\Delta \delta N^2)/5)^2]^{1/2}$$  \[1\]

Identification of the differences between the apo and ADP states was less straightforward
because the apo state was not assigned. It was evident from the titration spectra of ADP into the 
apo form that ADP was in slow exchange between both states so that it was not possible to track 
the peaks that changed positions between ADP and apo states. Nevertheless, many resonances 
remained unchanged, or the changes were less than the linewidth of the resonance. This allowed 
us to carry out the following, limited, mapping of chemical shift changes. We determined for 
every \(^1\)H-\(^{15}\)N TROSY resonance of the ADP form a resonance that was the closest in the TROSY 
spectrum of the apo form. In several cases, the resonance of the ADP state disappeared and no 
clear candidate new resonance was found in its vicinity in the apo form. For these cases we 
assumed that the resonance of the apo state has shifted away at least as far as the closest peak, 
even if that latter peak would normally be associated with another residue. This is justified 
because the shifted peak could be hidden under the latter peak, and that mere disappearance of 
the peak due to exchange broadening is not to be expected because the system is in complete 
slow exchange with no substantial broadening for any of the resonance peaks, even for those that 
resonate very closely in the two states.

This treatment of the data amounts to the quantitation of the minimal chemical shift 
changes per residue that must occur to explain the differences between the two NMR spectra. It 
is valid only when exchange broadening processes associated with the titration can be excluded, 
as is the case for our data. We enumerate the obtained minimal TROSY shift data, \(\Delta\delta_{\text{min}}(\text{NH})\), 
by adapting the usual equation: 

\[
\Delta\delta_{\text{min}}(\text{NH}) = \left( (\Delta\delta_{\text{min}} H^2 + (\Delta\delta_{\text{min}} N/5)^2)/2 \right)^{1/2}.
\]
Results

The isolated N-terminal nucleotide binding domains of *E. coli* DnaK and bovine Hsc70 form structural units that are capable of binding and hydrolyzing ATP (30-32). Sequence alignments indicated that the first 381 residues of TTK should form a homologous domain. The 44 kDa construct studied here included a 20 residue N-terminal leader sequence, MGSSHHHHHHSSGLVPRGSH, that incorporated a 6 residue His-Tag and a thrombin cleavage site followed by the residues 1-381 of TTK. Proteolytic removal of the leader sequence had no discernable effects on the spectra of the TTK-NBD domain (23).

Assignment of the ADP Bound Form of TTK NBD- All assignment spectra of the TTK-NBD were collected at 55 °C. A °H-15N TROSY spectrum of the TTK-NBD at 55 °C in the ADP-Pi bound state is shown Figure 2A and is consistent with a folded, monomeric protein. The assignment process, outlined in the Experimental Procedures, utilized a standard set of three dimensional, triple resonance NMR experiments collected on a sample of 2H, 15N, 13C labeled protein (23). Initial assignments were done on the protein in the ADP-Pi bound state. This process resulted in backbone assignments of 335 of the 362 (92%) non-proline residues in the sequence. None of the peaks of the N-terminal leader were assigned suggesting that it underwent chemical exchange on an intermediate timescale. The program CSI (33) was used to predict the secondary structure based on the backbone assignments. The predicted secondary structure closely matched that observed for the aligned residues in x-ray structures of Hsc70 thereby confirming the usefulness of a homology model for TTK based on the Hsc70 structures.

A noticeable feature of the ADP-Pi bound spectra is that several residues in different regions of the structure produce two sets of resonances. The relative intensities of these pairs of peaks were 1:1.1 +/- 0.2 (where the RMSD spread is due to limited signal/noise ratio) suggesting
that those locations on the protein were in an equilibrium between two conformations that had approximately equal populations. The two states could have been due to intramolecular rearrangements or intermolecular (monomer-dimer) interactions. To test whether the states were due to monomer-monomer interactions a series of TROSY spectra of the ADP form of TTK were collected at concentrations from 100 to 900 µM. The relative intensities of the pairs of doubled resonances remained constant over this concentration range establishing that the two conformations involved were a result of intramolecular rearrangements. Peaks separated by as little as 20 Hz could be resolved indicating that this exchange was occurring on a timescale longer than 50 msec; complete absence of line broadening on these peaks indicates that the exchange process is actually slower than 100 ms (kex < 10 s⁻¹).

**Nucleotide-Dependent Chemical Shift Changes between the apo and ADP states**-The TROSY spectrum of the apo form at 55 °C was also typical of that of a folded, monomeric protein of 44 kDa. A total of 365 strong resonances could be observed in this spectrum which was close to the expected number of 381 non-proline residues in this construct indicating that the protein was likely present in a single conformation. As described in the Experimental Procedures section, ADP was titrated into an apo sample and TROSY spectra were collected at several protein to nucleotide ratios. As increasing amounts of ADP were added, the intensity of many resonances decreased proportionally with the protein : nucleotide ratio while a similar number of new peaks appeared indicating that the nucleotide was in slow exchange with the protein. A slow nucleotide exchange rate is consistent with the published off-rate (0.67(+/−0.79 x 10⁻³ s⁻¹) for ADP for the full length TTK (19). Comparison of the TROSY spectra of the apo form with the fully saturated ADP form (Fig. 2B) showed that while more than 200 peaks were identical between the two spectra at least 150 other resonances had shifted more than a linewidth.
This would indicate that while a substantial portion of the molecule had identical structures in the ADP and apo states, more than a third of the residues had experienced some change in the local conformational environment.

Since the assignment sample of the ADP form had contained 10 times excess ADP, it was possible that some of the nucleotide induced changes were caused by secondary nucleotide-protein interactions. The $K_d$ reported for ADP binding to full length TTH DnaK is in the 50 to 280 nM range (19), therefore, assuming that nucleotide affinity is similar for the isolated NBD, the protein should be virtually saturated with nucleotide at the 1:1 ratio at the concentrations used in our experiments. Addition of ADP at ratios greater than 1:1 did not result in further spectral changes, confirming this expectation.

Chemical shifts can be perturbed by many factors including changes in covalent structure, alterations in average local three dimensional structure, or in electrostatic fields (34). The NH chemical shifts being observed in TROSY spectra are also very sensitive to changes in hydrogen bond strength, (34) that may not entail large scale structural differences. It is not possible to determine the type or magnitude of structural changes solely from chemical shift perturbation data. Peaks assigned to residues throughout all of the subdomains of TTK exhibited shifts upon the addition of ADP, suggesting widespread nucleotide induced structural differences of undetermined nature or magnitude. However, it is generally accepted that larger changes in chemical shift are correlated with more significant changes in structure. The total magnitude of change in chemical shift, $\Delta \delta(NH)$, is usually calculated using equation 1 to give equal weight to the shifts in each frequency dimension and to identify the residues undergoing the largest changes.

Since the NMR spectrum of the apo form of the protein was not assigned, it was not
possible to directly measure the magnitudes of the differences between the apo and ADP states. In this case the differences were estimated by the minimally required shift changes (MRSC) (see Experimental Procedures). Figure 3 shows the sequence distribution of the MRSC's and Figure 4A shows the mapping of the largest changes onto the model structure. The division of the NBD into subdomains 1A, 1B, 2A and 2B is illustrated in Figure 4A and follows those defined for the Hsc70 structure (31). Significant shifts occurred in all four of the subdomains with a large number surrounding the nucleotide binding site. Other changes were concentrated on both faces of the nucleotide binding cleft between subdomains 1B and 2B and in subdomain 1A.

Characterization of the ATP bound state—Addition of a saturating amount of ATP to the apo form of the NBD resulted in a TROSY spectrum distinct from the apo state but with a strong overall similarity to the ADP state. At a slight excess of excess ATP the TROSY spectrum showed only 15 significantly shifted peaks from the ADP state. It is also evident that the relative intensities of the doubled peaks that were observed in the ADP spectrum had changed in the ATP state while a larger number of other resonances had narrowed. An example of changes observed (Fig. 5A) shows two peaks assigned to two conformations accessible to Arg75 in TTK in the ADP form. Figure 5B and 5C, respectively, show the TROSY signals from Arg75 immediately after the addition of a five-fold excess of ATP to the apo form and after 40 hour incubation at 55 °C. As can be seen, the intensities of the two Arg75 peaks reverted to that of the ADP-bound state which we associate with the hydrolysis of ATP over this period. The entire ATP-bound spectrum reverted to that of the ADP state with a half-life of ~8 hours demonstrating that this domain was a functional ATPase. It was therefore not possible to obtain triple resonance spectra that take up to 72 hours to collect for assignment of the ATP state. ATP hydrolysis and the slow rates of nucleotide exchange results in a mixed ADP/ATP state, even in the presence of a large
excess of ATP.

A titration of ATP into the apo form of TTK, similar to that described previously with ADP, was performed to identify potential secondary nucleotide interaction sites. As expected for the slowly exchanging nucleotide the spectrum changed, dramatically with substoichiometric amounts of ATP. At nucleotide to protein ratios above 1:1 the NH peak of residue Thr185 was observed to undergo a progressive shift with increasing nucleotide concentration, indicating that a local fast exchange binding process occurred. Several of the residues close to Thr185 in the model structure also showed very small concentration dependent shifts at excess ATP. No other parts of the protein showed this type of change and no homologous change was observed during the ADP titration. Since Thr185 is located more than 20 Å from the nucleotide binding site, this change likely denotes a secondary, low affinity interaction that is not saturated at the 2:1 ratio. The nature of this interaction is not clear as the region around Thr185 in the model structure has negative charge due to the presence of 3 neighboring Glu residues that would seem to make interactions with the negatively charged ATP unlikely. Possibly the excess ATP competes for cations which may interact in solution with that negatively charged region.

To unambiguously observe the "ATP-bound" state on the timescale required for NMR experiments, it was necessary to use a non-hydrolyzable ATP analogue. Previous studies (8,35) had indicated that the ATP analogue AMP-PNP, was not useful in inducing the same conformation in E. coli DnaK as ATP binding. It has been reported that another commonly used ATP analogue ATPγS was not useful because the commercially available preparations contain up to 25% ADP (8). TROSY spectra of TTK in the presence of ATPγS (data not shown) produced a spectrum almost identical to that observed for the mixed ATP/ADP state suggesting that ADP contamination is a problem. As an alternative we added a mixture of aluminum and
fluoride salts that have been found to form complexes with ADP in the active sites of many ATP binding proteins (36,37). ADP-AlF₅ complexes mimic ATP or a transition state between the ATP and ADP forms. Addition of AlCl₃, NaF and ADP to a sample of the NBD resulted in a spectrum that was almost identical to that of the pure ATP form. Only 4 peaks were noticeably shifted between the ATP and ADP-AlF₅ spectra. The residues that differed included Thr185 and Glu184 that, as previously discussed, exhibited an ATP concentration dependent shift. The other 2 residues that differed were Glu28 and Gly206 which are located on widely separated surfaces of the protein. The most substantial difference between the spectra was observed for the almost 80 peaks that were broadened or doubled in the ADP form. In the ADP-AlF₅ form these peaks were found to have sharpened considerably or to have entirely shifted intensity to one peak. In the ATP state these peaks were intermediate between that seen in the ADP and ADP-AlF₅ spectra, likely a result of the mixture of bound ATP and bound ADP-Pi. The high degree of similarity between the ATP and ADP-AlF₅ spectra strongly suggests that the conformations are very similar.

Crystal structures of complexes of other proteins with ADP-AlF₅ have shown that the latter can adopt either an ADP-AlF₃ or ADP-AlF₄ form. Schlichting and Reinstein (37) found that crystallization conditions, particularly the pH, and the stereochemistry of the nucleotide binding site influence the fluoride coordination number and geometry of the ADP-AlF₅ complex. In the absence of a crystal structure of TTK we were not able to positively identify the AlF₅ state from our experiments, however, the pH of the NMR sample was 7.4 which would tend to favor the AlF₃ state.

Mapping Conformational differences between Nucleotide-bound states-The ADP-AlF₅ form of the protein was assigned independently using the three dimensional, triple resonance
NMR experiments described in Experimental Procedures. Using these experiments and with reference to the previous ADP state assignments the backbone resonances of 322 residues were assigned. These assignments allowed a quantitative analysis of the chemical shift difference between the nucleotide bound states. Figure 6 shows a histogram of $\Delta \delta (\text{NH})$ values between the ADP and ADP-AlFx states for each residue. It is apparent that the differences between the nucleotide bound states are much less widespread than those between the apo and ADP states shown in Figure 3. The average shift calculated was $0.089 \pm 0.37$ ppm. The residues that exhibited large shifts greater than 0.2 ppm are mapped onto the model structure in Figure 4B. A large proportion of the shifted residues (Lys3, Ala4, Thr133, Ala141, Asn144, Ala146, Asn167 and Glu168) are located at the interface between subdomains 1A and 1B while several of the others are located proximal to the nucleotide binding site.

As mentioned previously, the assignment of the ADP form established that almost 80 resonances exhibit either two resolved peaks of approximately equal intensity or a single peak with a somewhat broadened peak shape. These peaks were clearly identifiable in the ADP-AlFx spectrum to give a single or narrowed peak. All of these changes were in accord with the changes in the observed in the ATP spectrum but were more clearly defined in the ADP-AlFx spectrum. Figure 5D shows the two peaks of Arg75 in the ADP state shifting all intensity to one frequency in the ADP-AlFx state. The changes in the doubled and broadened peaks between the nucleotide states was a clear indication that the ADP form was in slow conformational exchange between two states while only one form was detectable in the presence of ATP (ADP-AlFx). Of the two ADP-bound states one was very similar to the ATP-bound form while the other was unique to the ADP bound state. Comparison of the apo and ADP-bound spectra showed that the both ADP substates were distinct from the apo form.
Discussion

The Apo State- The extent of the chemical shift perturbations between the apo and nucleotide bound forms of TTK was surprising in light of previous studies that suggest the apo and ADP states are functionally and structurally similar. The kinetics of peptide binding (38) and partial proteolytic digests (8) of the apo form of *E. coli* DnaK were found to be similar to the ADP form. The marked difference in the TROSY spectra, with an average minimal shift index of 0.03 ppm, between the apo and the ADP bound state was therefore unexpected, in particular since chemical shift mapping revealed that difference occurred throughout the NBD.

The apo state of the NBD had been characterized at atomic resolution in complex with the respective nucleotide exchange factors GrpE for *E. coli* DnaK (39) and a domain of Bag-1M for Hsc70 (40). In the case of these complexes, the NBD was found to be very similar to the structure of the free ADP and ATP states of Hsc70 with the exception of an opening up of the nucleotide binding cleft mostly by the movement of subdomain 2B. The differences in the clefts was attributed to the presence of the exchange factors. Our present data NMR suggest that widespread conformational differences of the apo state from the nucleotide bound states are concentrated around the nucleotide binding site, in subdomain 1A and between the faces of the nucleotide binding cleft, including subdomain 2B. We therefore suggest that the altered cleft opening also takes place in the apo form likely as an equilibrium in the absence of the exchange factors. The adenine ring of the nucleotide forms a part of the interface between subdomains 2A and 2B while the phosphates coordinate interactions from all four subdomains. In the absence of nucleotide there is likely more freedom for (dynamic) interdomain conformational
Comparison of ATP and ADP-AlF₃ States—There was a very high degree of similarity between the TROSY spectra of the ATP and ADP-AlF₃ forms. The most significant difference was the complete absence of the doubled peaks in the ADP-AlF₃ form that were partially present in the ATP form. Only 4 residues were found to have peaks that did not correspond with any peaks in the ATP form and 2 of those were in the immediate vicinity of the putative secondary low-affinity ATP/cation binding site around residue 185. We thus conclude that the interaction with this extraneous site occurs solely in the presence of ATP. The two remaining differences do not form a contiguous patch and are likely due to other nonspecific binding effects. We have not been able to directly determine, either by NMR or mass spectrometry, whether the bound ADP-AlF₃ condensate is in the AlF₃ or AlF₄ form. Both forms can represent a transition state analogue for the hydrolysis of ATP (the former as ADP-AlF₃.H₂O), while the ADP-AlF₃ form may also constitute a non-hydrolyzable ATP mimic. Because we were not able to discern clear differences between the NMR spectra of TTK-NBD in the ATP form and the ADP-AlF₃ form, it is most likely that ADP-AlF₃ is present as a non-hydrolyzable ATP mimic in the ADP-AlF₃ form. Nevertheless, the possibility that the structure of TTK in the presence of a transition state analogue form of ADP-AlF₃ condensate is nearly identical to that of the ATP-bound form, cannot be excluded.

Nucleotide Hydrolysis Induced Changes in TTK-NBD—The most salient difference between the NMR spectra of TTK-ADP and TTK-ATP (or TTK-ADP-AlF₃) is the presence of extensive NMR TROSY peak doubling and broadening for 80 residues in the former and none in the latter. For most instances, both corresponding peaks could be independently assigned using triple resonance connectivities. Generally the chemical shift differences between the
corresponding peaks are very small (0.01 ppm in Δδ(NH)) suggesting that changes in the local structure are very small. The relative intensities of the corresponding peaks are consistently 1:1.1 +/- 0.2 and the residues that display this behavior are widespread over the protein molecule (Fig. 5B). These combined observations strongly suggest that TTK-NBD.ADP is subject to a global two-state equilibrium with ΔG~0, for which the kinetics are slower than 10 s⁻¹.

Residues showing two conformations or broadening are mostly dispersed throughout subdomains 1A, 1B and 2A while subdomain 2B contain less. Of particular note is that two conformers were present for residues Leu254, Val58 and Lys84 that are located on the interior of the nucleotide binding cleft near the top of the molecule (indicated with arrows in Fig. 4B). These residues correspond to residues in E. coli DnaK that Bremher et al. (41) identified as forming salt bridges and hydrophobic contacts across the cleft. The observation that these residues exist in two states in the ADP form of TTK NBD suggests that the conformational changes involve a reorientation of the lobes of the nucleotide binding cleft, possibly an opening of the cleft. Opening and closing of the cleft has been suggested to be part of the mechanism of nucleotide exchange (42) and therefore this type of motion is not unexpected but interestingly in this case it seems to occur exclusively in the ADP form. Many of the residues that showed the largest chemical shift perturbation in their TROSY positions, when comparing the nucleotide states, are clustered at the interface between subdomain 1A and 1B (see Fig 4B). The undefined structural changes at the 1A-1B interface may effectively turn the region from a stable conformation in the ATP state to that of a hinge that allows the conformational exchange in the ADP-bound form.

The location of many of the perturbed and conformationally exchanging residues in subdomains 1A and 1B correlate strongly with the positions of residues identified in mutational
studies of *S. cerevisiae* Ssc1 (43) and *E. coli* DnaK that exhibited impaired *in vivo* chaperone activity (44). In both of those studies it was found that while those mutations did have some effect on the interdomain allostERIC coupling in the isolated DnaK, they had a much more dramatic effect on interactions with the cochaperone DnaJ (Hsp40). Consequently, this hinge region was described as a binding channel for DnaJ. Our data indicates that this putative DnaJ binding site coincides with an area of interdomain allostERIC interactions in the isolated DnaK NBD. The DnaK-DnaJ interaction results in a greatly enhanced chaperone activity over isolated DnaK and it is possible that DnaJ mediates the interdomain communication differently. Additionally, the location of many of the perturbed residues is also close to the region where the linker to the substrate binding domain is located in the full-length protein, and corresponds to an area that is sensitive to mutagenesis that interferes with the SBD-NBD allostERIC communication (M. P. Mayer, personal communication) Thus, in the full-length protein, the NMR-detected changes in this region could present a different interaction surface to the SBD and/or to DnaJ depending on the nucleotide state.

**Implications for the AllostERIC Mechanism**—AllostERIC control of substrate binding by the 44 kDa regulatory NBD is intrinsic to the function of the Hsp70s (45). The allostERIC coupling between substrate affinity and the nucleotide state requires that the NBD interact differently with the SBD in the presence of the two nucleotides. This change in the interdomain interaction has been presumed to be the result of a conformational change in the NBD upon nucleotide hydrolysis. Based on conventional ideas about allostery it would be expected that this change would involve a ligand-induced conformational switch from a "R" to a "T" state that would result in the rearrangement of the domains and/or the exposure/disruption of a hydrophobic/polar patch. Recently, a comparison of the SBD in the apo and peptide forms has revealed both
conformational and dynamic differences between the two states (46) that could be a source of interdomain allosteric communication. Partial proteolysis (8), crosslinking (35), small angle x-ray scattering (47,48) and fluorescence measurements (8,49) indicate that full-length Hsc70 proteins undergo a nucleotide-dependent global conformational change. It has therefore been surprising that comparison of several x-ray structures of the NBD of Hsc70 in both the ADP and ATP forms show them to be almost completely superimposable (see Fig. 1) (10).

An alternate allosteric mechanism has been proposed by Mayer et al (50), based mostly on kinetic data, in which E. coli DnaK, in both nucleotide states, is in a dynamic equilibrium between two conformations. One of the conformations would open the substrate binding site to allow fast binding and release of substrate while the other would close it. In this model ATP binding would simply alter the balance of the pre-existing dynamic equilibrium toward the open state and hence lower substrate affinity. Hydrolysis of ATP to ADP would change the equilibrium so that the SBD was more often in the closed conformation resulting in an overall tighter substrate binding. The observation, in this study, of a two-state conformational exchange in the ADP form between an ATP-like state and a second conformation provides support for a model of the high affinity state being a dynamic equilibrium between the open and closed forms. The fact that the conformational equilibrium between these states is slow (k<sub>ex</sub> < 10 s<sup>-1</sup> as seen from our NMR data, possibly much slower) is required by function, as it as would allow sufficient time for substrate binding to the open low-affinity state. The single conformation observed in the ATP-bound state suggests however that the population of the low affinity state is essentially completely in the open conformation. Interestingly, Farr et al (35) previously proposed a single conformation ATP state and two conformation ADP state for E. coli DnaK based on crosslinking data. In Figure 7 we put forward a model for the allosteric mechanism
similar to that of Farr et al. (35) but with the additional insight, based on NMR monitoring of changes on a per-residue basis, that one of the ADP states, termed D(L), is very similar to the low affinity ATP conformation (T(L)). This type of mechanism is consistent with a growing body of evidence that allosteric changes can frequently be represented as ligand-induced shifts in the populations of a set of dynamically interconverting substates (51-53).

The ability of the NBD to adopt the ATP-bound conformation in either nucleotide state may explain the lack of structural differences observed between the published crystal structures of the Hsc70 NBD in the ADP, ATP and AMP-PNP states. The crystal packing apparently favors the ATP conformation under the conditions used in those studies and drives the equilibrium in the ADP samples towards the ATP conformation.

This study has allowed the re-interpretation of much of the previous data on the allosteric mechanism from the molecular perspective to insight of conformational properties at the residue/atomic level. The observation of a significant nucleotide dependent change in the dynamics of the NBD has resulted in support for and refinement of the dynamic equilibrium model for the allosteric mechanism as well as identification of specific residues involved. Current work in our lab concentrates on further structural characterization of the observed differences in states.

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**Figure Legends**

Figure 1

(A): Overlay of backbone traces of 12 crystal structures of the NBD of Hsc70 with ADP-Pi or ATP or AMP-PNP bound and/or various single site mutations. The backbones almost completely superimpose with no visible structural changes between the different states. The PDB files used were 1ATS, 1ATR, 1HPM, 1KAX, 1KAY, 1KAZ, 1BAO, 1BA1, 1BUP, 2BUP, 1QQN and 3HSC.

Figure 2

(A): $^1$H$^{15}$N 800 MHz TROSY spectrum of the ADP bound form of TTK at 55 °C, 400 µM. (B):
Overlay of a region of the $^1$H$^{15}$N TROSY spectra of the ADP form (black) and apo form (blue) of TTK. More than 150 residues exhibited chemical shift changes between the apo and ADP bound forms. (C): Superposition of the same region of the ADP bound spectrum (black) with the ADP-AlF$_x$ bound spectrum (green) that is highly homologous to the ATP state. Only 14 residues showed significant changes in chemical shift between these forms. An additional 80 residues gave evidence of a change from 2 conformations in slow exchange in the ADP-state to a single state in the presence of the ATP analogue.

Figure 3:
Histogram of minimal required chemical shift changes (see Experimental Procedures) between the ADP-Pi and apo states of TTK by residue number. The largest MRSC values ($\Delta\delta_{\min} (\text{NH}) > 0.03$ ppm) are plotted on the model structure of the TTK NBD in Figure 4A.

Figure 4:
(A) Homology model of the NBD of with *T. thermophilus* DnaK color-coded to indicate the position of residues that changed their NMR resonance position upon ADP binding to the apo form. The gray ribbon indicates the positions of residues that did not show chemical shifts or that were unassigned. Red indicates positions of residues that did shift significantly ($\Delta\delta_{\min} (\text{NH}) > 0.03$ ppm). The ligand, ADP-Pi is shown in green. The structural subdomains discussed in the text are indicated on this structure. (B) The positions of residues with chemical shift differences between the ADP and ADP-AlF$_x$ forms greater than 0.2 ppm are indicated by red ribbon. The sites on the ribbon colored yellow are indicative of residues in slow two-state conformational
exchange in the ADP form but not in the ATP form. The arrow indicates the residues K54, V58, and E256 on both sides of the nucleotide binding cleft (see text) that were observed to be in slow exchange between conformations. The ellipse identifies the interface between subdomains 1A and 1B where the largest chemical shift changes were observed.

Figure 5:
Changes in the signal from Arg 75 in the different nucleotide states observed in TROSY experiments. (A) The split resonance of Arg 75 in the ADP state. (B) The signal from Arg 75 observed in the presence of an excess of ATP. Note that some of the intensity has shifted from the downfield to the upfield position. (C) The resonance of Arg75 observed 40 hours after the addition of ATP. Note about half of the peak intensity has returned to the downfield peak. (D) Arg 75 in the presence of ADP-AIF₆. Note that all of the intensity is now in the upfield position.

Figure 6:
Histograms of the backbone amide group chemical shift differences, δ(NH), for TTK between the ADP-Pi and ADP-AIF₆ states, (A) residues 1-199, (B) residues 200-381.

Figure 7:
Proposed model for the allosteric mechanism of DnaK based on the dynamic equilibrium observed in this report. T(L) refers to the low affinity ATP bound state, D(L) and D(H) refer to low and high affinity states respectively that are in a dynamic equilibrium in the ADP bound state. T(L) and D(L) are very similar in structure. This model closely parallels one proposed by Farr et al. (35).
Revington et al Fig. 4B
\[ D(H) \]

\[ D(L) \]

\[ T(L) \]

\[ D(L) \approx T(L) \]
NMR study of nucleotide-induced changes in the nucleotide binding domain of Thermus Thermophilus Hsp70 chaperone DnaK: Implications for the allosteric mechanism

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