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Allostery in the Hsp70 chaperones is transduced by subdomain rotations

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SUMMARY

Hsp70s are central to protein folding, refolding, and trafficking in organisms ranging from *Archae* to *Homo sapiens*, both at normal and at stressed cellular conditions. Hsp70s are comprised of a nucleotide-binding domain (NBD), and a substrate-binding domain (SBD). The nucleotide binding site in the NBD and the substrate binding site in the SBD are allosterically linked: ADP binding promotes substrate binding, while ATP binding promotes substrate release. Hsp70s have been linked to inhibition of apoptosis, i.e., cancer, and diseases associated with protein misfolding such as Alzheimer's, Parkinson's, and Huntington's.

It has long been a goal to characterize the nature of the allosteric coupling in these proteins. However, earlier studies of the isolated NBD could not show any difference in overall conformation between the ATP and ADP state. Hence the question: how is the state of the nucleotide communicated between NBD and SBD?

Here we report a solution NMR study of the 44 kDa NBD of the Hsp70 from *Thermus thermophilus*, in the ADP and AMP-PNP states. Using the solution NMR methods of residual dipolar coupling analysis, we determine that significant rotations occur for the different subdomains of the NBD upon exchange of nucleotide. These rotations modulate the access to the nucleotide binding cleft, in absence of a nucleotide exchange factor. Moreover, the rotations cause a change in accessibility of a hydrophobic surface cleft remote from the nucleotide binding site, which previously has been identified as essential to the allosteric communication between NBD and SBD. We propose that it is this change in the NBD surface cleft that constitutes the allosteric signal that can be recognized by the SBD.

INTRODUCTION

Hsp70s (heat shock 70 kDa) chaperone proteins are central to protein folding, refolding, and trafficking in organisms ranging from *Archae* to *Homo Sapiens*, both at normal and at stressed cellular conditions¹. The Hsp70's recognize misfolded substrate proteins by their exposed

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Supplementary material:

PDB-format coordinate files for the NBD of DnaK T. *Thermophilus* in the ADP and AMPPNP state. The PDB files also list the RDCs used. A data sheet containing nine figures and one table.

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hydrophobic amino acids. In a process that requires ATP and two co-chaperones², the Hsp70's unfold the substrate proteins by cycles of binding and release. The unfolded proteins then refold on their own accord. Much of the mechanism by which this takes place remains unknown. Recently, Hsp70s have been linked to cancer^{3; 4; 5; 6} and diseases associated with protein misfolding such as Alzheimer's⁷, Parkinson's⁸, and Huntington's^{9, 10}. It has been suggested that modulation of Hsp70 activity with small compounds may form an avenue to treat these diseases¹¹.

Hsp70s are comprised of two main domains: a 44 kDa N-terminal nucleotide-binding domain (NBD), and a 25 kDa substrate-binding domain (SBD) that harbors the substrate-binding site. The Hsp70's are allosteric molecules: with ADP bound to the NBD, substrate binds tightly to the SBD ($K_D=1.5 \times 10^{-7}$ M); ATP binding to the NBD promotes substrate release from the SBD ($K_D=4.6 \times 10^{-5}$ M)^{12, 13}.

While several high-resolution crystal and solution structures are available for the NBD^{14; 15; 16} and SBD^{17; 18; 19; 20; 21; 22} subdomains of the Hsp70's of several species, no high-resolution structure for a complete wild-type Hsp70 has been reported. Hence, the allosteric mechanism is not yet known. However, several recent studies have shown that a conserved, hydrophobic and flexible linker (residues³⁸⁸VQDLLLLDVTP (Hsc70 *H. sapiens*),³⁸⁰VRDVLLDVTP (DnaK *T. thermophilus*)), loosely tethers the NBD and SBD in the ADP-substrate-bound state^{23; 24; 25}. In addition, it is anticipated that NBD and SBD are docked together in the ATP state^{24; 26}, but this still needs to be demonstrated for the Hsp70s with an actual structure. Currently, the following overall model for the allosteric mechanism seems credible. With substrate bound in the SBD and ADP.Pi in the NBD, the SBD collides repeatedly with the NBD, but does not dock²⁵. Upon ATP binding to the NBD, a change takes place in the NBD such that the collisions lead to stable docking of SBD and NBD. The docking event propagates through the SBD and leads to release of substrate. A key feature of this model is that the NBD conformational change upon ADP $\rightarrow$ ATP change takes place while the SBD is loosely tethered. Hence, it is expected that the isolated NBD should also show such changes between different nucleotide states.

However, this notion is seriously challenged by X-ray diffraction studies of the isolated NBD. They show no obvious difference in overall conformation of the NBD of bovine (identical to human) Hsc70 between the ATP and ADP state^{14; 27} (see Figure 1) in the crystalline state. In addition, small-angle X-ray scattering methods could not detect a difference in the radius of gyration for the ATP and ADP state of the isolated NBD of Hsc70 in solution either²⁸, apparently supporting the crystallographic studies.

We report here that subtle but extensive chemical shift changes occur in the NMR spectra of the isolated NBD of Hsc70 between the ADP.Pi and ATP state in neutral aqueous solution (see Fig. 2a). Clearly, change in nucleotide causes changes in shifts that extend far from the nucleotide site. This, indeed, suggests that the isolated NBD changes its conformation upon nucleotide change, in the absence of the SBD. The question is: what is the extent of the change? In order to answer this question, we changed the focus of study to the NBD of the Hsp70 homologue DnaK of *Thermos Thermophilus*²⁹ (TTh-NBD), because the collection of NMR data at elevated temperature (50 °C) allows better quality spectra than for the mesophilic Hsc70. TTh-NBD is highly related to human Hsc70 (49% identity, 72% homology). As is shown in Fig. 2b, quite similar chemical shift changes occur in the NMR spectrum of TTh-NBD in the ADP and AMP-PNP states, where the latter is a non-hydrolysable ATP analogue.

In this work, we use residual dipolar coupling analysis^{30, 31}, and discover that subdomain IIB in this species (see Fig. 1) rotates up by as much as 20 degrees between the AMP-PNP and ADP state. Such conformational changes have been observed before, but were thought to be

caused by the binding of a nucleotide exchange factor; such as BAG¹⁶ or GrpE³², rather than by nucleotide change itself. We also discover that subdomains IA and IIA rotate significantly with respect to each other, opening (AMPPNP) and closing (ADP) a hydrophobic surface cleft between these subdomains. Significantly, this cleft area has been hypothesized to be of importance to the communication between NBD and SBD^{23; 24}. The current work demonstrates that a change in this cleft occurs upon nucleotide exchange. It is likely that this is *the* allosteric change on the surface of the NBD that is recognized by the SBD and/or the hydrophobic linker between NBD and SBD.

RESULTS

The NBD's of the Hsp70s are comprised of four subdomains¹⁴ called IA, IB, IIA and IIB, as defined in Figure 1. The nucleotide, ATP or ADP.PO₄, together with several cations, is located deep in the central cleft and interacts with all four subdomains. AMPPNP binds at the same location. Several crystal and some NMR structures are available for several domains of Hsp70's of several species (called DnaK in *bacteria* and SS1 in *funghi*) molecules, but no *de-novo* structure is available for any domain of the DnaK of *Thermos Thermophilus*.

We used the construct³³ of TTh-NBD containing residues 1-381, which gives rise to excellent ¹⁵N-¹H HSQC-TROSY NMR spectra at 50 °C (see supplementary materials). The assignments of the backbone resonances for this construct were obtained previously³³. The NMR spectra of TTh-NBD in the ADP.PO₄ and AMPPNP state show many chemical shift differences (Figure 2b). The amide ¹H and ¹⁵N NMR assignments were confirmed for each state by analysis of 3D HNCA-TROSY spectra. The shift changes are subtle but significant, as is shown in Figure 3. Mapping the shift differences on a TTh-NBD homology model, shows that the shifts extend far beyond the location of the nucleotide (see Figure 2b). The shifts disclose subtle but widespread changes in structure and/or dynamics between the states.

At 44 kDa, TTh-NBD is too large for a high-resolution structure determination by NMR. However, the reorganization of subdomains can be probed, with excellent precision, using residual dipolar coupling NMR (RDC) analysis in solution³¹. Briefly, the method is as follows. The target protein is aligned in neutral aqueous buffer to which Pf1 phage is added³⁴. The rod-like phages align in the magnetic field, providing an anisotropic environment for the protein occupying the buffer between the phage molecules. The target protein aligns, dynamically, about 0.2% of the time, allowing the magnetic dipolar coupling of the amide nitrogen and hydrogen nuclei to be measured by solution NMR techniques^{35, 36}. We use modified HSQC-TROSY experiments that shift the resonances in the ¹⁵N dimension proportionally to ¹J_{NH} + ¹D_{NH}. The comparison of those spectra with unshifted TROSY spectra reveals the residual dipolar couplings³⁵ (see supplementary materials) This information is, in turn, used to obtain the orientation of the protein's sub-domains with respect to the magnetic field, making use of the available high-resolution (X-ray) structures and homology models of the sub-domains. Subsequently, this information is used to reconstruct the relative orientation of the sub-domains. Sub-domain orientation is characterized by three axes corresponding to a rectangular parallelepiped with the longest axis defined as S_{zz}, the shortest as S_{xx} with S_{yy} in between.

On average, 60 reproducible NH RDC's per subdomain were obtained, with an uncertainty of ~4 Hz. Only RDCs corresponding to residues that are homologous between the NBD's of DnaK of *T. thermophilus*, DnaK of *E. coli*, and Hsc70, Hsp70 and mt-Hsp70 of *H. sapiens* were used. This reduced the number of RDCs available for analysis to about 50 per subdomain (see Table 1). We used a homology model for a reference structure of the subdomains (see Materials and Methods).

As is shown in Figure 3, our data is of sufficient quality define, with precision, orientations of the different subdomains for the two nucleotide states. In these so-called Sanson-Flamsted globe plots, the main orientational axis S_{zz} for all subdomains is located approximately at 40 °West, 5 °South. This average location by itself is irrelevant, as it just reports the difference between the physical S_{zz} axis orientation of the aligned protein and the arbitrary PDB-file Z-axis direction of the model structure used. However, the differences between the globe locations of the S_{zz} axes of the different subdomains are highly relevant: they indicate that not all subdomains are oriented as in the model structure. Moreover, the relative orientations of the subdomains change between the ADP and AMPPNP state. The spread in the S_{zz} directions of the subdomains represents the uncertainty in the raw data as determined by the Monte-Carlo routine in the REDCAT program used, using RDC's corresponding to homologous positions without any further editing. According to the REDCAT program, for the AMPPNP state the subdomains are, within experimental error, oriented similarly to those in the model structure, which is based on bovine Hsc70 in the ADP state. Surprisingly, this is not the case for the ADP state, where the orientation of subdomain IIB has moved away by approximately 20 degrees. In addition, the relative orientations of the other subdomains change as well.

As a next step in the analysis, the orientational changes were modeled as changes in the 3D structure. As explained in detail in the materials and methods, the models for the ADP and AMPPNP states were obtained by superposing the rotated subdomains on the model structure for minimal positional RMSD using translation of coordinates only.

Comparison of the best-fit structures based on all RDC's (as defined in the legend of Table 1) are shown in Figure 4. Major shifts are seen for the relative orientations of all domains between the two nucleotide states.

In order to assess the statistical significance of the differences seen in Figure 4, we performed a "Jack-Knife" self-validation procedure. As shown in the Materials and Methods section, it was determined that the calculation of model structures obtained from RDC calculations in which 40% of the RDC data was deleted at random, yields an ensemble that represents a reliable measure of the precision of the structure determination.

One of the ensembles is presented in Figures 5, in which subdomains IA are superimposed. The figures clearly show that the average position of subdomain IIB in the ADP state (relative to the orientation of IA) lies outside of the ensemble of possible subdomain orientations in the AMPPNP state. Figure S6 shows the reverse is also true. Hence, the differences in orientation between these states as seen in Figure 4 are statistically highly significant. The average position of subdomain IB in the ADP state lies well inside the ensemble of possible subdomain IB positions in the AMPPNP state. Hence, the differences in orientation between these states as seen in Figure 4 are statistically NOT significant. The average position of subdomain IIA in the ADP state lies at the boundary of the ensemble of possible subdomain positions in the AMPPNP state. And *vice-versa*. According to T-statistics, there is just a 3 % probability that the observed difference in orientations of subdomains IIA in the different nucleotide states is by chance (see Materials and Methods). Hence, the statistical significance of the differences for subdomains IIA and IIB as seen in Figure 4 is high.

Summarizing, our data shows that the subdomains in the AMP-PNP state are, within experimental error, similarly oriented as in the model structure, which is based on an Hsc70 NBD crystal structure in the ADP state (3HSC.pdb, ref ¹⁴). In contrast, in the ADP state, one observes statistically significant deviations, by as much as 20 °, for the S_{zz} axes of subdomains IIB. In addition we observe statistically significant rotation of subdomain IIA with respect to subdomain IA between the different states, as disclosed in Figure 4.

DISCUSSION

The RDC data reports on the orientations and changes in orientations of the NBD subdomains. The data gives no information whether the subdomains shift in a lateral way with respect to each other or not. Therefore, it is important to emphasize that the structures shown in the figures are just convenient models; they show the orientational changes, but changes in translation have been minimized by superposition. The models have not been further minimized, and contain areas in which atoms clash (see also Materials and Methods).

Figure 4 shows that domain IIB rotates about 20 degrees clockwise going from AMP-PNP to ADP.Pi state. As was shown in Figure 5, this change is significant within the accuracy of the experimental data. This movement is the likely explanation for the chemical shift changes for residues in domains IB and IIB that line the nucleotide binding cleft (see Figure 2). We suggest that the origin of this rotation is as follows. In the AMP-PNP state, the left and right halves of the protein are bridged by the rigid nucleotide mimic. For ADP.Pi, however, the γ -phosphate bond is hydrolyzed, which breaks the molecule and thus the bridge between left and right, allowing a rotation of IIB. It appears that the left and right halves can move relatively independently in the ADP.Pi state. Possibly, the ADP.Pi structure can open dynamically even further than displayed in the models.

Available crystal structures of the isolated NBD correspond closely to each other regardless their nucleotide state. This is illustrated in Figure 1, which is an overlay of five crystal structures of the NBD of bovine Hsc70 in APO, ADP (twice), ADP-V₂O₅ state and the mutant K71M complexed with ATP from two different laboratories. Small Angle X-ray Scattering (SAXS) measurements³⁷ showed no significant difference in radii of gyration for the ADP and ATP states of the human Hsc70 NBD fragment either (see Table 2). This appears to confirm that the conformations of these states are also identical in solution. However, there is also no difference in the computed radii of gyration for the AMPPNP and ADP structures of TTh-NBD as derived from the RDC data, despite the rather large (visual) differences (Table 2).

As it turns out, the X-ray structures of the isolated NBD all correspond much closer to the AMPPNP state of Tth-NBD in solution than to the solution ADP state (Figure 6a). The fit between Tth-NBD on the ADP state with HSC70 in ADP state is inferior (Figure 6b), mostly because of the clockwise rotation of IIB. The rotation of subdomain IIB in the ADP state in solution is very reminiscent of the rotation of this domain upon binding of a nucleotide exchange factor (NEF). Figure 6c shows a superposition the crystal structures of bovine Hsc70-NBD with and without its NEF BAG-1¹⁶. Similar rotations were observed for *E.coli* DnaK NBD upon binding to its NEF GrpE³⁸ (not shown) and in the very recent co-crystal structures of yeast Hsp70 NBD with Hsp110 as exchange factor^{39, 40} (not shown). Hence, the rotated state that exists in solution in for the ADP state for DnaK Tth-NDB, can also be observed in the crystal, provided it is stabilized by a NEF.

However, earlier notions that it is the interaction between Hsp70 and NEF that forces an induced fit which actively promotes nucleotide exchange³², should likely be modified. It seems more appropriate to assume that the NEF selectively captures and stabilizes the ADP state. The nucleotide exchange process in the Hsp70 chaperones (abundant ATP replacing ADP) in this model is then catalyzed by other processes, which may include the interaction of GrpE's tail with the SBD, as has been suggested before³⁸. Selective interaction of the NEF with the ADP conformation also makes sense from a functional point of view: it avoids unnecessary interference with the ATP state and provides a driving force for NEF release when the nucleotide has been exchanged from ADP to ATP. The process of selective capture which we here suggest to be active for the chaperone NEF interaction, has recently been recognized as an common interaction paradigm for several other allosteric proteins⁴¹ and nucleic acids⁴².

The chemical shift changes in Figure 2 suggest that major changes take place in domains IA and IIA when changing from the AMP-PNP to the ADP.Pi state. The globe plots in Figure 3 and the models based on this data in Figure 4 suggest that these changes are due to a clock wise rotation of domain IIA with respect to domain IA when going from AMPPNP to ADP state. Figure 5 proves that such a rotation is significant with a 97 % probability.

This is the first time that such a conformational change has been demonstrated for nucleotide change in a single Hsp70 species. But, as is illustrated in Figure 7, similar differences are present between the published structures of *different* species in different nucleotide states. For instance, a crystal structure for a yeast Hsp110 dimer, locked in the ATP state, was recently solved²⁶. Hsp110 has been recognized as a protein with a strong Hsp70 homology, but it functions as a NEF rather than as a chaperone. It was suggested that the structure of this related protein might provide insight in the structure of an Hsp70 protein in the ATP state. If one associates the structure of Hsc70 NBD in complex with its NEF as a *true* representative of the ADP state, and Hsp110 as a *true* representative of the ADP state, we can ADP - ATP change becomes quite apparent. Figure 7a shows a dramatic clockwise rotation of domain IIA of Hsc70 (complexed with BAG) as compared to Hsp110 structure. Remarkably, these *intermolecular* ADP-ATP changes are very similar to the *intramolecular* changes seen for TTh-NBD as shown in Figure 4 (which is reproduced for convenience in Figure 7b). That conformational changes are possible in the Hsc70 NBD, has been suggested by us before⁴³. However, our experimental data at the time was of insufficient quality to disclose what the changes entailed.

At this point of the discussion it is worthwhile to take stock. Our studies have shown that significant conformational changes occur in the nucleotide binding domain of DnaK of *Thermus thermophilus* upon nucleotide change. This is confirmed by and does explain the observed chemical shift changes for this domain in its different states. It is of importance that similar chemical shift changes were observed between the ADP and ATP state of the Hsc70 NBD; confirming that the similar conformational changes take place for the different species. Analysis of the RDC data and subsequent modeling, discloses significant and substantial clockwise rotations for domains IIA and IIB when changing from AMPPNP state to ADP state (relative to IA). Because of these findings, we can be now also confident that the differences seen between structures of different species in different nucleotide states are most likely due to changes in nucleotide, and not to changes in species. Hence, those differences may now be interpreted in terms of conformational changes and the mechanism of allostery. The remainder of this discussion is a first attempt.

The relative rotations of subdomains IA and IIA are of great interest, since it affects a hydrophobic surface cleft between them (see Figure 8), which likely is involved in linker binding, and substrate domain binding^{23; 24}. In Hsp110, this surface cleft is open and is occupied by the linker between the NBD and SBD. The cleft is closed in the ADP state and the linker cannot be docked. It has been shown before for DnaK of *E.coli* that the linker moves freely in the ADP state and allows the SBD to move relatively freely^{24, 25}, while the linker becomes immobilized in the ATP state²⁴. Several biochemical studies also showed that the linker is exposed in the ADP state, and not in the ATP state^{23, 44, 45}. It has been speculated²⁴ that changes in the surface cleft between IA and IIA are responsible for the docking in the ATP state, but it is only in our current work that this is substantiated with an actual observation of change in that area for a single species.

On the basis of the current work, literature reports on a confounding large number of Hsp70 species, states, complexes and artificial truncations, studied by different experimental techniques, can now be merged into the following general allosteric model: in the ATP-substrate-free state, NBD and SBD are docked. The NBD is relatively rigid^{44, 46}. ATP hydrolysis leads to a loosening of the junction between the “left” (subdomains IA and IB) and

“right” (subdomains IIA and IIB) of the NBD. Overall, the NBD has become more flexible^{44; 46}. In this process, subdomain IIA rotates clockwise and closes the IA/IIA surface cleft. Next, the linker is expelled and the SBD dissociates from the NBD. We suggest that the ATP hydrolysis energy is spent on this step to compensate for the lost SBD-NBD binding energy. In the SBD, the dissociation from the NBD is transduced through the beta sheet²¹, and affects the substrate-binding cleft, which rigidifies. Meanwhile, subdomain IIB rotates clockwise as well, and is predisposed to bind to the NEF, which further stabilizes the open, ADP state and promotes nucleotide exchange. The re-binding of ATP causes NBD and SBD to dock, and expels the substrate. We suggest that the regained SBD-NBD docking energy compensates for the lost SBD-substrate binding energy.

While this scenario is likely to be correct overall, many of the details are still missing. For instance, we don’t really know how the NBD/SBD linker binds in the ATP state for the true Hsp70’s. The Hsp110 model²⁶ is useful, but there is only marginal sequence homology: true Hsp70’s have a fully hydrophobic linker such as ³⁸⁸VQDLLLLDVTP (Hsc70 *H. sapiens*), ³⁸⁰VRDVVLLDVTP (DnaK *T. thermophilus*), ³⁸⁷TQDILLLDVAP (*Candida albicans* SSA-1) while yeast Hsp110 has ³⁸⁹VRPFKFEDIH. Moreover, Hsp110 is locked in the ATP state, and the divergent linker may actually be the cause of its inability to change conformation.

Being fully aware of the limits on the precision of current NMR structure calculations and models, we tentatively show in Figure 8 a comparison of the IA/IIA cleft region for the ADP and AMPPNP state of DnaK *T. thermophilus*. The surface residues visible on this side of the protein are completely conserved in over 300 sequences of archaea, bacteria and animals checked. The hydrophobic nature of the cleft is clear. Access to the cleft becomes hindered in the ADP state. This is especially obvious for Leu 174, rendered in cyan, which forms the bottom of the cleft. In the TROSY spectrum of NBD-Tth, L174 shows a large shift upon nucleotide exchange. Its position is also indicated in Figure 2b. The homologous residue, I181 in Hsc70, also shifts (Fig. 2a). NMR chemical shifts were also observed for this residue (L177) in DnaK of *E. coli* upon nucleotide exchange²⁴.

Mutagenesis studies²³ strongly suggest that the universally conserved R167 of DnaK *E. coli* interacts with the universally conserved D393 of the linker sequence in the ATP state. The NMR shift data in Figure 2 and the surface view in Figure 8 shows that this residue is unaffected by nucleotide exchange (R164 and R171 in DnaK *Tth.* and Hsc *B. taurus*, respectively). This is not surprising since the constructs studied here are truncated before the linker sequence. However, the finding suggests that this Arginine residue is “passive” and just serves to steer the sense of linker insertion. This sense corresponds to the insertion of the linker in Hsp110²⁶.

MATERIALS AND METHODS

Samples

DnaK-Tth 1–381 (DnaK-Tth NBD), cloned into Pet22-b (Novagen) with an N-terminal His-tag, was expressed in *E. coli* BL21 cells at 37 °C. Expression was induced at O.D. 600 = 0.5 in a triple labeled M9 medium containing 98% D₂O, ¹³C-glucose and ¹⁵N ammonium chloride. Cells were harvested by spinning down at 15,000 g, were resuspended and subsequently lysed using a microfluidizer. The protein was purified in two steps using Ni-NTA agarose with an imidazole gradient and FastFlo Q- ion exchange at pH 7.2 with a linear KCl gradient⁴⁶. The purified protein was extensively dialyzed against NMR buffer (see below) and concentrated using Centricon micro filters.

NMR experiments

NMR samples contained 0.15–0.2 mM protein in 50 mM HEPES, pH 7.4, 10 mM KCl, 5 mM MgCl₂, and 5 mM sodium phosphate. ADP or AMP-PNP concentration was 10 mM. Experiments were performed at 50 °C on an 800 MHz Varian Inova spectrometer, using a triple resonance cold-probe. Backbone resonance assignments were obtained from a single 3D HNCA-TROSY experiment for each nucleotide state, using a previously obtained peak list for DnaK-Tth in the ADP-AlF_x state as a template³³. 281 and 310 assignments were obtained for the ADP and AMP-PNP form, respectively.

For RDC measurements, Pf1 bacteriophage in the aforementioned buffer was added to the NMR samples to a concentration of 20 mg/ml for partial alignment⁴⁷. The ²H₂O quadrupolar splitting was 8 Hz at these circumstances. RDCs were extracted from a series of 2D TROSY experiments with a [kt1/2-180(N,H)-kt1/2] sequence⁴⁸ at the beginning of the ¹⁵N chemical shift labeling period, with $\kappa=0$, 0.75 and 1.5. This method is better suited for larger proteins than the IP/AP analysis⁴⁹ or the TROSY/HSQC comparison⁵⁰, since this method retains the full TROSY narrowing in the ¹H dimension and partial TROSY narrowing in the ¹⁵N-dimension. The 2D NMR spectra were recorded at 50 °C. 10, 20 and 40 hours of data acquisition was used for the $\kappa=0$, 0.75 and 1.5 experiments, respectively, for both the ADP and AMPPNP state.

Data analysis

The experiments yield TROSY spectra, in which the ¹⁵N coordinate of each cross peak is shifted by $\kappa \cdot ({}^1J_{\text{NH}} + {}^1D_{\text{NH}})/2$ (see Figure S2). Using a uniform value of ¹J_{NH} 90.5 Hz, the ¹D_{NH} was extracted from the differences between the $\kappa=0$ and $\kappa=0.75$ and between the $\kappa=0$ and $\kappa=1.5$ spectra. The expected differences in real ¹J_{NH} (± 2 Hz) for the different residues is significantly less than the experimental precision in the data (± 4 Hz in the ¹⁵N dimension) for this large protein.

The available RDC data for each state was trimmed down in two successive steps. First, the RDC's obtained from the differences between the $\kappa=0$ and $\kappa=0.75$ and between the $\kappa=0$ and $\kappa=1.5$ spectra were compared. Those for which the scaled differences were larger than the scaled RMS difference between these values, were rejected. The scaled differences were defined as $\text{difference} = \text{abs}(2 \cdot (\text{value1} - \text{value2}) / (\text{value1} + \text{value2}))$. The remaining RDC's were labeled as “experimentally sound”. Second, only the RDCs were retained for NH groups of residues that are homologous between the following Hsp70's: DnaK of *T. Thermophilus*, DnaK of *E.coli*, Hsc70, Hsp70, mt-Hsp70 and Bip of *H. sapiens*.

The remaining RDC data, termed experimentally and homology sound, was used as input to orient subdomain IA, IB, IIA and IIB of the DnaK-Tth homology model. It was assumed that each subdomain was a rigid unit by itself. While most of the data shown in this paper is based on this relatively unedited RDC input data, several calculations were performed with a subset of RDC data in which only those residues for which RDC data was available in both the ADP and AMPPNP state were retained. The results of these calculations, shown in Figure 7b, shows that the differences in conformations is essentially the same as for the larger RDC dataset, establishing that the differences are not due to the selection of the RDCs.

We used in-house written programs and REDCAT⁵¹ (A Residual Dipolar Coupling Analysis Tool) to transform the RDCs to the orientational data. REDCAT's solution algorithm relies on singular value decomposition and Monte Carlo error estimation to generate an ensemble of 1000 structures compatible with the input structures and the set of RDCs provided, based on an experimental error range of 4 Hz. The in-house written Fortran program is based on a grid and minimization search of all possible Euler rotations, overall alignment and rhombicity, to

find the best fit to the experimental data. The results are identical to the REDCAT solutions. The structural data shown in this report were all derived from the results of Fortran program, since it gave easier access to the distribution of Euler angles.

The orientations of the subdomains was computed from the structures of the subdomains. Since no coordinates are available for NBD-Tth, we used a homology reference model. It was constructed in two steps. First, we threaded the sequence of Tth-NBD on the coordinates of a crystal structure of the NBD of DnaK-*E. Coli*, the protein most homologous to DnaK *T. thermophilus* for which coordinates are available³² (1DKG.pdb, with a resolution of 2.8 Å). However, this structure was obtained in the presence of a nucleotide exchange factor, which likely rearranged the subdomain orientations. Several crystal structures¹⁴ without co-chaperones are available for the bovine Hsc70 NBD, in which the subdomain orientations, especially for IIB, are different from that seen in 1DKG.pdb. In order to obtain the required reference model for NBD-TTh, we adjusted the orientations of the subdomains in the model to correspond with those in 3HSC.pdb, a structure of Hsc70-NBD nominally in the ADP state at a resolution of 1.90 Å. The adjustment was based on a superposition of the Ca atoms of the secondary structure elements in the subdomains. The quality of the superposition of the TTh-NBD reference model with 3HSC.pdb can be seen in Figure S6.

Self validation

An alternative estimation of the significance of the differences observed in Figure 5 can be provided by a “Jack-Knife” self-validation procedure. In self-validation, a certain fraction of the data is omitted at random, and the remaining data is then fitted to the relevant mathematical model. This process is carried out many times, and a distribution in fitting parameters is obtained. In order to assess the relationship of the self-validation data retention percentage, the distribution in the fitting parameters and the real error, we carried out the following model calculation. To a 3rd order polynomial function, random noise was added to an amplitude that allowed the data to be fitted back to a 3rd polynomial with a R^2 of 0.65 (see Figure S7). This was repeated ten times to generate ten independent rather noisy “data sets”. The distribution in the ten sets of fitted parameters was taken as a measure of the achievable precision due to the “real” noise in the “data” (see Table S1). Subsequently, one of the “data sets” was analyzed using self validation. When 60% of this single “data set” is kept at random and fitted back to a 3rd polynomial, and doing this ten times, one ends up with a similar distribution in the ten sets of fitted parameters as for the “real” noise (see Table S1). Hence, we view self validation retaining 60% of the data as a realistic measure of the influence of measurement error on the precision of the fitted parameters.

Figure S8 shows the results of 100 self validations per domain using 60% of the RDC data, selected at random, computed with the REDCAT program. Even when using only 60% of the data, one obtains statistically significant differences in sub domain orientations as compared to the model structure.

Molecular models

We obtained the molecular models for each of the states in five steps:

1. Rotate each subdomain into its Principle Axes System, (PAS) based on the average Euler angles obtained from the 60% retained self validation procedure (obtaining IA-PAS, IIA-PAS, IB-PAS, IIA-PAS)
2. Rotate the reference structure into the PAS of domain IA (TTh_{REF}^{PAS-IA})
3. Optimize the superposition of the Ca's in the 2nd structure of each PAS subdomain onto the corresponding subdomain of TTh_{REF}^{PAS-IA} , using translation only

4. Reassemble the domains into a single structure
5. Check that this process has not altered the relative subdomain orientations by carrying out a REDCAT computation on the obtained models. Zero degree Euler angles should be and were obtained.

The obtained model structures are available in pdb format in the supplementary materials.

The model of TTh-NBD in the AMPPNP state was used as a template for all super positions of structures shown. Corresponding CA atom positions in secondary structure elements of subdomain IA were used for all overlays.

Ensemble of RDC structures

Using the in-house written Fortran programs, we calculated self-validations at the 60% level (retained) for an ensemble of 30 structures for the ADP state and for the AMPPNP state (see Table 1). The RMSD's of the three Euler angles were computed from these ensembles as shown in Table 1. Next, for each subdomain we calculated a molecular structure, as described above, for each of the 8 different sets of Euler angles: $\langle\alpha\rangle\pm\text{RMSD}^\alpha$, $\langle\beta\rangle\pm\text{RMSD}^\beta$ and $\langle\gamma\rangle\pm\text{RMSD}^\gamma$ as shown in Table 1.

Statistics of the ensemble

The 30 structures in the ensembles are represented by 8 structures describing the boundaries of the ensemble, like the 8 vertices of a cube. These boundaries represent the average RMSD multiplied by $\sqrt{3}$. While the ensemble contains 30 structures, it should actually be seen as ensemble of just 6 independent structures, since it takes 5 RDC's to determine an orientation (the 4-fold degeneracy in orientation is not an issue here). Under the assumption that this distribution of 6 structures can be described as a Gaussian around an average, the T-statistic can be used.

In the present case, the average orientation of subdomain IIA in the ADP state lies at the edge of a similar ensemble of that domain in the AMPPNP state, and *vice versa* (Figure 6). The T-statistic for similar ensembles, is defined as (http://en.wikipedia.org/wiki/Student%27s_t-test)

$$t_{1,2} = \frac{\langle X_1 \rangle - \langle X_2 \rangle}{\sigma_{X_1 X_2} \sqrt{\frac{2}{N}}}$$

where $\langle X_1 \rangle$ and $\langle X_2 \rangle$ are the averages of the ensembles, where N is the number of elements in each ensemble (the same), and where

$$\sigma_{X_1 X_2} = \sqrt{\frac{\sigma_{X_1}^2 + \sigma_{X_2}^2}{2}},$$

with $\sigma_{X_1}^2$ ($\cong \sigma_{X_2}^2$) as the squared standard deviation of the distributions.

For the present case, with $\langle X_1 \rangle - \langle X_2 \rangle = \sigma_{X_1 X_2} \cdot \sqrt{3}$ and $N=6$ one finds $t_{1,2}=3$, which means that there is just a 3% probability that the observed difference in orientations for subdomains IIA between the two states is by chance (http://changingminds.org/explanations/research/analysis/t-test_table.htm).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Stereograph of the superposition of five X-ray crystallography structures for bovine Hsc70 NBD (4–380). The proteins were crystallized in the following states: wt-APO (2QW9.pdb, chain A), wt-ADP.PO₄ (3HSC.pdb and 2QWL.pdb, chain A), wt-ADP.VO₄ (2QWM.pdb, chain A) and K71M-ATP (1KAX.pdb). The proteins were overlaid on the secondary structure elements of sub domain IA (Hsc70-count residues 1–39, 116–188, 361–381; blue) to accentuate potential changes in sub domains IB (residues 40–115; green), IIA (residues 189–228, 307–360; purple) and IIB (residues 229–306; cyan). The N-terminus is in red, the C-terminus of this domain is shown in yellow. The nucleotide, PO₄³⁻, Mg²⁺ and two Na⁺ present in 3HSC.pdb are shown in space fill. The figure was prepared with MacPyMOL.

Figure 2.

A: ^{15}N - ^1H chemical shift differences between the ATP and ADP.Pi conformation of Hsc-70-NBD. Orange: significant shift, green: no shift, grey: not known. Residues R171 (R167 in DnaK *E. coli*) and I181 (L177 in DnaK *E. coli*) are rendered as sticks.

B: ^{15}N - ^1H chemical shift differences ($\Delta\delta$) between the AMPPNP and ADP.Pi conformation of TTh-NBD. Red: $\Delta\delta > 2\sigma_{\Delta\delta}$; Orange: $\sigma_{\Delta\delta} < \Delta\delta < 2\sigma_{\Delta\delta}$; yellow: $0.5\sigma_{\Delta\delta} < \Delta\delta < \sigma_{\Delta\delta}$; green: $\Delta\delta < 0.5\sigma_{\Delta\delta}$; grey: not known, where $\Delta\delta = (\Delta\delta_{\text{H}}^2 + \Delta\delta_{\text{N}}^2)^{1/2}$. ADP is in light blue, PO_4^{3-} in dark blue. Residues R164 (R167 in DnaK *E. coli*) and L174 (L177 in DnaK *E. coli*) are rendered as sticks. See also Figure 3. The figure was prepared with MacPyMOL.

Figure 3.

Globe graphs (Sanson-Flamsteed plots) showing the orientations and experimental uncertainties of the S_{zz} principal alignment axes (around 60 °West and 5 °South) of the different subdomains of NBD of DnaK-Tth as derived from the NMR RDC measurements using REDCAT⁵¹. A and B: RDC input: conserved residues and experimentally reproducible A (left) AMP-PNP state; B (right) ADP.Pi state.

Color code: IA (1–37, 109–180, 357–377; blue); IB (residues 38–108; green); IIA (residues 181–219, 307–356; purple) and IIB (residues 220–304; cyan). The reference structure for the NBD of DnaK-Tth was modeled on the NBD of DnaK *E. coli* (1DKG.pdb) and bovine Hsc70.ADP.Pi (3HSC.pdb).

Scale: Horizontal: 20 ° per gridline, vertical, 10 ° per gridline. (The S_{zz} principal alignment axes also appear at the other side of the globe (120 °East, and 5 °North). The S_{xx} axes appear as smears around 150 ° West, 45 ° North and 30 °East, 45 °South).

Figure 4. Model of the ADP state of the NBD of DnaK T.Th (RED) superposed on domain IA of the model for the AMPPNP state (BLUE) calculated using all RDCs

Figure 5.
The average of the Tth-NBD self-validation ensemble for AMPPNP (blue) and Tth-NBD ADP (red) compared to the self-validation ensemble of Tth-NBD ADP (salmon)

Figure 6.

Comparison of structures. The figures were made by superposing the CA-positions of the secondary structure elements of domains IA.

A: Hsc70 bovine (3HSC.pdb, green) and NBD-TTh with AMPPNP (blue)

B: Hsc70 bovine and NBD-TTh with ADP (red)

C: Hsc70 bovine and Hsc70 bovine complexed with BAG (1HX1.pdb, orange; BAG is not shown)

The figure was prepared with MacPyMOL.

Figure 7.

Comparison of structures. The figures were made by superposing the CA-positions of the secondary structure elements of domains IA.

A: Hsc70-bovine complexed with BAG (1HX1.pdb; orange; BAG not shown) and Hsp110. (1QXL.pdb; NBD of one monomer shown, in brown).

B: DnaK-TTh-NBD-model in the AMPPNP state (blue) and DnaK-TTh-NBD-model in the ADP state (red). The figure was prepared with MacPyMOL.

Figure 8.

View of the IA / IIA interface of DnaK-TTh. This view is a 90 degree rotation around the horizontal axis as compared to the other figures. It shows the “bottom” as compared to these other figures.

A: in the ADP state. B: in the AMPPNP state

Color coding:

In domain IA (left) hydrophobics, light-green; positive, light-blue; negative, purple; polar, grey
In domain IIA (right) hydrophobics, green; positive, blue; negative, red; polar, white The C-terminus (residue 372 in this construct) is rendered in cyan. Domain IB (at left top) is in grey; domain IIB (at right top) is in black.

RDC calculations for the NBD of DnaK of *T. thermophilus* in 50 mM HEPES, 10 mM KCl, 5 mM MgCl₂, and 5 mM sodium phosphate, 20 mg/ml Pfl phase, pH 7.2, at 50 °C. ADP or AMP-PNP concentration was 10 mM.

Table 1

Input RDC ^a	N _{RDC} ^b	α	β	γ	S _{xx}	S _{yy}	S _{zz}	RMSD ^c Qd
		(°)	(°)	(°)				Hz
AMPPNP state								
IA common ^e	45	161.8	87.6	63.1	8.6E-04	2.1E-05	8.1	0.65
IA all ^f	58	161.2	86.9	61.8	8.1E-04	6.6E-05	9.8	0.73
IA selfval average ^g	34	160.2	87.4	61.9	8.6E-04	5.7E-05	9.1	0.68
IA selfval RMSD ^g	4	6.2	4.5	5.2	6.3E-05	7.4E-05	1.6	0.10
IB common	26	136.4	76.4	70.0	7.5E-04	3.1E-04	10.5	0.67
IB all	37	143.3	81.5	66.8	7.0E-04	1.5E-04	11.4	0.76
IB selfval average	23	145.3	81.4	66.2	7.4E-04	1.6E-04	11.1	0.74
IB selfval RMSD	3	26.3	5.3	4.5	9.3E-05	8.6E-05	0.9	0.06
IIA common	24	163.0	90.6	61.1	5.8E-04	2.6E-04	7.2	0.60
IIA all	45	145.6	87.5	59.7	7.2E-04	1.9E-04	7.3	0.56
IIA selfval average	27	144.6	88.0	58.7	7.4E-04	1.9E-04	7.0	0.53
IIA selfval RMSD	4	6.9	5.9	4.6	6.4E-05	8.9E-05	0.6	0.06
IIB common	34	159.8	90.3	56.9	1.0E-03	4.0E-05	7.6	0.54
IIB all	45	165.1	92.2	66.1	8.0E-04	-1.7E-05	9.2	0.71
IIB selfval average	28	166.7	92.9	69.8	8.1E-04	1.0E-06	9.0	0.71
IIB selfval RMSD	4	7.0	3.7	10.0	6.6E-05	3.1E-05	1.2	0.10
ADP.Pi state								
IA common	45	150.4	85.5	67.4	8.7E-04	1.9E-04	8.0	0.59
IA all	66	155.3	86.7	68.3	6.8E-04	1.7E-04	8.8	0.70
IA selfval average	41	154.4	87.1	68.2	6.9E-04	1.9E-04	8.5	0.67
IA selfval RMSD	3	12.3	5.3	4.8	9.5E-05	7.6E-05	0.6	0.05
IB common	26	115.6	82.1	70.3	7.3E-04	2.2E-04	8.4	0.58
IB all	38	107.2	85.2	71.4	6.0E-04	3.5E-04	9.0	0.66
IB selfval average	24	165.4	86.7	71.8	6.7E-04	3.1E-04	8.5	0.62
IB selfval RMSD	4	65.9	6.8	4.2	8.0E-05	7.0E-05	1.3	0.08

Input RDC ^d	N _{RDC} ^b	α	β	γ	S _{zz}	S _{yy}	S _{xx}	RMSD ^c Qd	
		(°)	(°)	(°)				Hz	
IIA common	24	180.5	78.2	66.2	-9.3E-04	6.1E-04	3.2E-04	5.5	0.43
IIA all	43	175.8	83.4	72.1	-1.0E-03	7.4E-04	2.7E-04	6.7	0.47
IIA selfval average	26	177.1	83.3	71.1	-1.0E-03	7.6E-04	2.4E-04	6.2	0.45
IIA selfval RMSD	4	8.7	3.6	4.8	5.4E-05	6.1E-05	6.0E-05	0.6	0.05
IIB common	34	161.5	87.5	50.1	-1.1E-03	1.1E-03	9.0E-05	7.9	0.51
IIB all	50	159.1	80.1	48.4	-8.7E-04	8.7E-04	-1.5E-06	8.3	0.60
IIB selfval average	30	156.2	79.9	50.1	-8.6E-04	8.5E-04	5.7E-06	7.9	0.58
IIB selfval RMSD	4	7.6	5.2	6.3	5.6E-05	7.7E-05	6.4E-05	1.0	0.06

The calculations were carried out using an in-house written grid search and optimization program, which optimized α , β and γ (Euler rotational angles in the z-y-z convention describing the orientation of the subdomain in the aligned protein with respect to the coordinates of the reference structure based on DnaK *E. coli* and Hsc70 *H. sapiens*), the overall alignment (S_{zz}) and the rhombicity ((S_{yy}-S_{xx})/S_{zz}).

^aThe input RDC set used

^bNumber of RDC's in that set

^cRMSD of the best fit between experimental and calculated RDC

$$Q = \frac{RMSD}{\sqrt{\frac{1}{N_{RDC}} \sum_{i=1}^{N_{RDC}} (RDC(i)_{exp})^2}}$$

^eConsists of experimentally reproducible RDC's, for NH groups of residues that are homologous between the following Hsp70's: DnaK of *T. Thermophilus*, DnaK of *E.coli*, Hsc70, Hsp70, mt-Msp70 and Bip of *H. sapiens*. This set was further reduced by taking only those residues for which RDC data was available in both the ADP and AMPPNP state.

^fConsists of experimentally reproducible RDC's, for NH groups of residues that are homologous between the following Hsp70's: DnaK of *T. Thermophilus*, DnaK of *E.coli*, Hsc70, Hsp70, mt-Msp70 and Bip of *H. sapiens*.

^g40% of the RDC's of (f) were omitted at random for an ensemble of 30 calculations per subdomain. The average and RMSD of the fitting parameters for these ensembles are given.

Table 2

Computed and experimental radii of gyration (Å)

	ADP	AMPPNP	ATP
Computed values for DnaK-TTh-NBD	21.55	21.63	
Experimental values for Hsc70-NBD Ref 37, Table 1	22.8 ± 0.2		22.6 ± 0.1
Experimental values for Hsc70-NBD Ref 37, Table 2	23.1 ± 0.1		

Computed and experimental radii of gyration. The experimental radii of gyration were taken from ³⁷