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Conformational Equilibria in Allosteric Control of Hsp70 Chaperones

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SUMMARY

Heat-shock proteins of 70 kDa (Hsp70s) are vital for all of life, notably important in protein folding. Hsp70s use ATP binding and hydrolysis at a nucleotide-binding domain (NBD) to control the binding and release of client polypeptides at a substrate-binding domain (SBD); however, the mechanistic basis for this allostery has been elusive. Here, we first characterize biochemical properties of selected domain-interface mutants in bacterial Hsp70 DnaK. We then develop a theoretical model for allosteric equilibria among Hsp70 conformational states to explain the observations: a restraining state, Hsp70_R-ATP, restricts ATP hydrolysis and binds peptides poorly; whereas a stimulating state, Hsp70_S-ATP, hydrolyzes ATP rapidly and has high intrinsic substrate affinity but rapid binding kinetics. We support this model for allosteric regulation with DnaK structures obtained in the postulated stimulating state S, with biochemical tests of the S-state interface, and with improved peptide-binding-site definition in an R-state structure.

Keywords

Allosteric regulation; crystal structure; DnaK; Hsp70; molecular chaperone; protein folding

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AUTHOR CONTRIBUTIONS

W.W., Q.L. (Qinglian) and W.A.H. designed experiments and analyzed data; Q.L. (Qinglian) and W.W. performed biochemical experiments; W.W. analyzed crystal structures, W.A.H. developed the theoretical analysis; Q.L. (Qun) built the hypothetical S-state atomic model and analyzed diffraction data; W.W. and W.A.H. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

INTRODUCTION

Heat shock proteins of 70 kDa (Hsp70s) are ubiquitous molecular chaperones that feature in diverse cellular processes. Going beyond namesake stress responses, they are crucial in normal cells for protein folding, disaggregation and degradation, for assembly processes, and for membrane translocation (Kim *et al.*, 2013); and, in disease, they are complicit for cancers (Calderwood & Gong, 2016) and protective against neurodegeneration (Ciechanover & Kwon, 2017). Hsp70s are preeminent players in protein homeostasis, feeding into Hsp60, Hsp90, Hsp100 and ubiquitination pathways (Rosenzweig *et al.*, 2019). Although many Hsp70s are stress-induced, others are expressed constitutively from essential genes (Daugaard *et al.*, 2007).

Hsp70 proteins act in ATP-dependent cycles of binding and release of client substrates, typically exposed hydrophobic segments in non-native polypeptides. ATP binds and is hydrolyzed at the nucleotide-binding domain (NBD) and client polypeptides associate at the substrate-binding domain (SBD). NBD and SBD are separable, but Hsp70 chaperone activity requires direct, albeit transient, allosteric interactions between these sites as linked together (Mayer & Gierasch, 2019). ATP binding to NBD dramatically decreases SBD affinity for client peptides, accelerating both on and off rates relative to ADP or apo states (Schmid *et al.*, 1994; Slepnev & Witt, 2002). Reciprocally, peptide binding stimulates ATP hydrolysis, after which bound peptides exchange slowly. Hsp40s stimulate ATP hydrolysis by Hsp70s and help to target them to substrates, and nucleotide exchange factors facilitate ADP release for the rebinding of ATP; nevertheless, the Hsp70 chaperone cycle proceeds self-sufficiently without these cofactors.

Crystal structures of individual NBD and SBD domains of Hsp70s provide a framework for biochemical understanding of Hsp70 chaperone activity. The prototype NBD structure is that from bovine Hsc70 (Flaherty *et al.*, 1990). It comprises four subdomains (IA, IB, IIA and IIB) built up from two structurally similar lobes (I and II), and it binds adenosine nucleotides at the interface between lobes with contacts from all four subdomains. The prototype SBD structure is from DnaK, an Hsp70 of *Escherichia coli* (Zhu *et al.*, 1996). Its compact β -sandwich subdomain (SBD β) is followed by an α -helical subdomain (SBD α), and it binds the NR heptapeptide (NRLLLTG) through a channel defined by loops from SBD β and covered by SBD α . NBD and SBD are flexibly linked in ADP or nucleotide-free states (Bertelsen *et al.*, 2009). Numerous biochemical analyses and additional x-ray and NMR studies gave insights into allosteric control in Hsp70s (Mayer & Kityk, 2015), showing NBD-SBD coupling when ATP is present; however, structures showing *bona fide* NBD-SBD interactions were elusive. A breakthrough came when Hsp110s, which bind ATP without hydrolysis, were shown to be evolutionary vestiges of Hsp70s (Liu & Hendrickson, 2007). Subsequent structures of *E. coli* Hsp70 DnaK with ATP (Kityk *et al.*, 2012; Qi *et al.*, 2013), identified here as DnaK_R-ATP, confirmed the dramatic conformational changes in SBD and inter-lobe shifts in NBD found in yeast Hsp110 Sse1.

The interactions between Hsp70 binding sites for ATP and polypeptide substrates clearly meet the literal definition of allostery. Yet, the elegant allosteric theories of Monod *et al.* (MWC, 1965) and of Koshland *et al.* (KMF, 1966) explain cooperativity between binding

sites in symmetric oligomers, as for hemoglobin; whereas Hsp70s function as monomers composed from linked binding domains for distinct ligands, making the particular treatments of MWC and KMF inapplicable. The concept that conformational equilibria between alternative states can govern the coupling interactions does apply however. Allosteric principles have been generalized in diverse studies (Cui & Karplus, 2008; Hilser *et al.*, 2012; Thirumalai *et al.*, 2019), but there are no prior quantitative treatments to address the allostery of Hsp70 proteins.

In this study, we first analyze biochemical properties *in vitro* for selected DnaK domain-interface mutants tested previously *in vivo* for the yeast Ssa1 and *E. coli* DnaK Hsp70s (Liu & Hendrickson, 2007). Biochemical properties of the mutant proteins pose unexpected puzzles. Thus, we develop a theoretical model for equilibria among Hsp70 conformational states to explain allosteric control of the chaperone activities. The theory explains observations on ATP hydrolysis and polypeptide binding from wild-type and mutant variant DnaKs by postulating conformational equilibria between a restraining state, Hsp70_R-ATP, which has a low rate of ATP hydrolysis and little or no peptide-binding affinity, and a stimulating state, Hsp70_S-ATP, which hydrolyzes ATP rapidly and binds client peptides with intrinsically high affinity but rapid on and off kinetics. This allosteric theory fits our biochemical results quantitatively. We then designed DnaK constructs to capture the postulated stimulating state and to improve our picture of the opened restraining-state SBD β . Finally, we use interface mutants to test the stimulating-state conformation and corroborate the allosteric theory.

RESULTS

Characterization of mutant variants at restraining R-state interfaces

Based on the Hsp110 structure of yeast Sse1, we previously tested homologous Hsp70 interfacial contacts using assays of cell viability and specific aggregation in response to heat shock. We found, in common for yeast Ssa1 and *E. coli* DnaK, that 8 of 9 mutated sites produced severe phenotypes (Liu & Hendrickson, 2007). Here, to assess these defects, we selected four profoundly defective and seemingly incisive mutations for *in vitro* biochemical characterization in full-length DnaK (Fig. 1A). The point mutants comprise N170D and V389D from the NBD-linker interface, I483D from NBD-SBD β , and I160D from NBD-SBD α , thus representing the three interfacial regions (Fig. 1B). All but N170 have exposed side chains in uncoupled domain structures. We also constructed the WT::NR fusion protein to present peptide NRLLLTG constitutively at the polypeptide binding site (Fig. 1C). These mutants expressed and purified as well as WT DnaK (Fig. S1A & B).

We tested each DnaK variant in multiple assays of allosteric interaction. Using a tryptophan-fluorescence assay (Palleros *et al.*, 1992; Buchberger *et al.*, 1995), we first checked for interdomain coupling. Fluorescence from W102, the only tryptophan in WT DnaK, was quenched and blue shifted (from 342nm to 335nm) when measured in ATP as compared with ADP – changes explained by hydrophobic contacts from SBD α in DnaK-ATP structures (Figs. 1B and S2A); whereas, none of the mutants showed fluorescence shifted in ATP versus ADP (Figs. 1D, S2B–H), indicating helix α A/B displacement for each mutant. We also tested 10 other mutations at I483 besides I483D (Fig. S3A), finding that impairment

of viability correlates inversely with quenching and blue-shifts as compared to wild-type (Fig. S3B); and we tested the cell viability of other I160 variants (Fig. S3C).

In a second set of assays, we examined the effects of peptide substrates on the rate of ATP hydrolysis (Figs. 1E, S4A–D; Table S1). As reported previously (Flynn *et al.*, 1989; McCarty *et al.*, 1995), hydrolysis is very slow in the absence of substrates (0.032 min^{-1} at 30°C for WT DnaK), but it accelerates markedly when peptides are present (up to 170-fold). To our surprise, most of the mutants showed highly elevated ATP hydrolysis even at low peptide concentration; only linker mutant V389D retained low intrinsic ATP hydrolysis. Indeed, the high basal rates for several of the mutant proteins made measurement difficult, so we turned to 20°C experiments where hydrolysis is slowed (basal 0.012 min^{-1} for WT; 0.37 min^{-1} for N170D, 31-fold enhanced).

In our third tests of allosteric interaction, we examined effects of ATP on the binding of peptide substrates (Figs. 1F, 1G and S4E; Table S2). It is well established that peptide substrates bind less tightly to Hsp70s in the presence of ATP than in its absence or in ADP (Mayer *et al.*, 2000). When in ADP, as expected for a dislodged and unperturbed SBD, we found that the interface-mutant proteins each bound the NR peptide with essentially the same affinity (K_D values of $1.49 \pm 0.10 \mu\text{M}$ to $1.59 \pm 0.08 \mu\text{M}$) as for WT DnaK ($K_D = 1.64 \pm 0.08 \mu\text{M}$). WT::NR did not bind free NR at all, consistent with a fully occupied SBD binding site. When in ATP, WT DnaK bound NR much less well than in ADP (apparent K_D , $K_D^{\text{APP}} = 37 \pm 5 \mu\text{M}$); whereas, in striking contrast, the mutant proteins bound NR tightly when in ATP. K_D^{APP} values in ATP ($1.73 \mu\text{M}$ average except for I160D at $2.91 \mu\text{M}$) were like those in ADP ($1.54 \mu\text{M}$ average). Nevertheless, despite having similar peptide affinities in ATP and ADP, binding kinetics for these mutants are distinctly faster in ATP than in ADP (Table S2), as for WT DnaK (Schmid *et al.*, 1994).

In light of the theoretical model developed below, we ascribe the structural interfaces tested here to be those in the predominating restraining state, DnaK_R-ATP (Fig. 1B).

Theory of allosteric equilibria in Hsp70 chaperones

How can we rationalize the observed biochemical characteristics? Clues come from findings on rates of ATP hydrolysis by various SBD constructs of DnaK. The ATPase activity of NBD truncated at linker residues 385 or 388, before the conserved 388DVLLLD₃₉₃ segment, is even lower than for basal WT DnaK; conversely, as here for I160D, N370D and I483D, constructs truncated at 392 or 393 showed constitutively high ATPase activities, 9-fold (Swain *et al.*, 2007) and 40-fold (Vogel *et al.*, 2006) enhanced over basal WT, respectively. NBD-linker interactions are evident when ATP is bound (Zhuravleva & Gierasch, 2011), and linker mutations disrupt the ATP enhancement (Vogel *et al.*, 2006), as we see here for V389D. Linker engagement alone suffices to potentiate the high rate of ATP hydrolysis observed for the linker-preserving SBD constructs. Whereas the prevailing picture has it that polypeptide substrates stimulate ATP hydrolysis, we suggest from the mutational analysis that the action of peptides may be described better as a release of inhibitory restraints. Thus, we postulate the existence of a stimulating ATP state, DnaK_S-ATP, that hydrolyzes ATP rapidly once NBD is freed of inhibitory interactions imposed by SBD in the intrinsically predominant restraining ATP state, DnaK_R-ATP.

In formulating a theoretical model, we designate the restraining ATP state as R, the stimulating ATP state as S, and the ADP or nucleotide-free states as U for uncoupled; the corresponding peptide complexes are RP, SP, and UP, respectively. We then postulate equilibria and hydrolysis rates to inter-relate these states (Fig. 2A). The equilibria are defined by the conformational equilibrium constant $K_{eqS} = [R] / [S]$ and intrinsic dissociation constants of $K_D^{OS} = [P] [S] / [SP]$ for the stimulating state and $K_D^{OR} = [P] [R] / [RP]$ for the restraining state. We assume that R and RP both hydrolyze ATP with an intrinsic basal rate of k^0 and that both S and SP hydrolyze ATP at the rate k' . This theory is elaborated fully elsewhere (Hendrickson, 2020), and we condense it here to the most salient aspects.

We first consider the impact of peptide binding on ATP hydrolysis, wherein the apparent catalytic rate constant k_{cat} expected in our single turnover experiment is the weighted average of hydrolysis reactions from all components:

$$k_{cat} = \{[R]k^0 + [RP]k^0 + [S]k' + [SP]k'\} / C_T \quad (1)$$

where C_T is the total concentration of all ATP states. As the reaction progresses, $C_T(t)$ decreases but rapid equilibration between ATP states will be expected to leave the relative abundances unaffected; thus, independent of time course, we obtain the result:

$$K_{cat} = \frac{[P](K' + (K_D^{OS} / K_D^{OR})K_{eqS}K^0) + K_D^{OS}(K_{eqS}K^0 + k')}{[P](1 + (K_D^{OS} / K_D^{OR})K_{eqS}) + K_D^{OS}(K_{eqS} + 1)}. \quad (2)$$

For the case of no binding to the restraining state ($K_D^{OR} = \infty$),

$$k_{cat} = \frac{k'[P] + K_D^{OS}(K_{eqS}K^0 + k')}{[P] + K_D^{OS}(K_{eqS} + 1)} = \frac{a[P] + b}{[P] + d}. \quad (3)$$

We then consider the impact of ATP binding on peptide binding. Neither the R and S states nor the RP and RS states are differentiated in a typical peptide binding experiment, whereby the apparent dissociation constant in the presence of ATP is $K_D^{APP} = [P] ([R]+[S]) / ([RP]+[SP])$. Consequently, apparent and intrinsic dissociation constants are related by

$$K_D^{APP}(ATP) = \frac{K_D^{OR} K_D^{OS} (K_{eqS} + 1)}{K_{eqS} K_D^{OS} + K_D^{OR}}. \quad (4)$$

For the limit of $K_D^{OR} = \infty$, i.e. no restraining state binding of peptides,

$$K_D^{APP}(ATP) = K_D^{OS} (K_{eqS} + 1). \quad (5)$$

When the allosteric equilibrium is at the restraining or stimulating state extreme ($K_{eqS} = \infty$ or 0, respectively), $K_D^{APP}(ATP) = K_D^{OR}$ or K_D^{OS} , respectively.

The simplified case of Eq. 5 has been described in the context of studies on an adenylate kinase (Schrack *et al.*, 2009), but we are not aware of previous formulations comparable to Eqs. 2 and 3 nor of any such theory for Hsp70s.

Biochemical tests of the allosteric theory

We made accurate measurements of ATP hydrolysis by WT and mutant variant DnaK proteins, and these data are fitted very well by Eq. 3 (Fig. 2B, Table S1). The fitted value of $k' = a = 0.276 \pm 0.012 \text{ min}^{-1}$ for the rate of stimulated hydrolysis by WT DnaK at 20°C agrees remarkably well with k_{cat} measurements from I483D ($0.271 \pm 0.011 \text{ min}^{-1}$) and from the WT::NR fusion protein ($0.273 \pm 0.008 \text{ min}^{-1}$), for which we calculate a local peptide concentration of 6.8 mM at the binding site (Zhou, 2001). Both are constitutively stimulated states ($K_{\text{eqS}} = 0$), whereby $k' = k_{\text{cat}}$. Although the hydrolysis data define k' uniquely, other parameters are degenerate as constrained by $d = K_D^{\text{OS}} (K_{\text{eqS}} + 1)$ and $b = K_D^{\text{OS}} (K_{\text{eqS}} k^0 + k')$. This degeneracy is broken by defining K_D^{OS} experimentally: $K_D^{\text{OS}} (\text{WT}) \equiv <K_D^{\text{App}} (\text{I483D})$, $K_D^{\text{App}} (\text{N170D}) > = 1.73 \pm 0.20 \text{ }\mu\text{M}$ since neither mutated site should affect peptide binding. Thereby, given the fitted values of $d = 115 \pm 9 \text{ }\mu\text{M}$ and $b = 1.33 \pm 0.14 \text{ }\mu\text{M min}^{-1}$, $K_{\text{eqS}} = 65 \pm 5$ and $k^0 = 0.0075 \pm 0.0020 \text{ min}^{-1}$. The S-state fraction is then $1/(K_{\text{eqS}} + 1) = 1.5\%$ in absence of client peptides, increasing with peptide concentration.

Peptide binding measurements in the presence of ATP are also compatible with the allosteric theory. As discussed above, fully stimulated mutant variants N170D and I483D are expected to bind client peptides in ATP with WT intrinsic S-state affinity ($K_D^{\text{OS}} = 1.73 \text{ }\mu\text{M}$), as they do with U-state affinity in ADP (Fig. 1F). Given this measure for K_D^{OS} and $K_D^{\text{App}} = 37 \text{ }\mu\text{M}$ as measured for WT DnaK-ATP, we obtain $K_D^{\text{OR}} = 53 \text{ }\mu\text{M}$ from a rearranged Eq. 4. Because the hydrolysis fittings imply no R-state binding ($K_D^{\text{OR}} = \infty$), we also devised an alternative explanation based on binding to a quasi-intermediate state Q in equilibrium with R and S (Hendrickson, 2020).

The reported structures of DnaK-ATP (PDBids 4b9q & 4jne) correspond to the R state, and the ‘allosterically active’ state described by Zhuravleva *et al.* (2012) likely corresponds to the S state. The conformational hint of this latter study is not definitive however. Neither the isoleucine methyl-TROSY NMR (Zhuravleva *et al.*, 2012) nor double electron-electron resonance (DEER, Lai *et al.*, 2017) experiments provide comprehensive structural detail, and differing conclusions were deduced from them about the NBD-SBD interface (each incompatible with x-ray structures described below). Moreover, by the theory, the S state is not a consequence of peptide binding; rather, peptide binding is a corollary of the S conformation; indeed, the S state is constitutive for I483D (Fig. 1E) and D481A (Kityk *et al.*, 2015), independent of client peptide.

Structural tests of the allosteric theory

The theoretical model predicts an alternative S-state conformation for ATP-bound Hsp70. What then is the nature of this stimulating state? We had biochemical clues: Constructs that delete SBD but retain the NBD-SBD linker hydrolyze ATP similarly as peptide-stimulated WT DnaK (Vogel *et al.*, 2006; Swain *et al.*, 2007); thus, we expected the NBD-linker in DnaK_S-ATP to be structurally similar to that in DnaK_R-ATP (PDBid 4jne). The K_D s

for binding of various peptides to various binding-site mutants (Mayer *et al.*, 2000) correlate well for DnaK in ATP versus ADP, and EPR studies found similar peptide-binding geometries for DnaK in ATP and ADP (Popp *et al.*, 2005); thus, we expected the SBD in DnaK_S-ATP to be like that in DnaK_U-peptide complexes (Zhu *et al.*, 1996; Bertelsen *et al.*, 2009). Peptide-binding kinetics are affected strongly by ATP (Schmid *et al.*, 1994; Table S2); thus, DnaK_S-ATP must have a communicative NBD-SBD interface. Fittings of theoretical alternatives to hydrolysis data showed that the S and SP states hydrolyze ATP indistinguishably (Hendrickson, 2020); thus, their NDB-SBD interfaces must be similar.

We first built a model with SBD_U re-oriented by 134° from DnaK_R-ATP (Liu & Hendrickson, 2013), but attempts at mutational validation were unresponsive. Then, knowing that S is distinguished from R by its facility for binding peptides and anticipating flexibility in peptide-free SBD, we focused on achieving the SP state as representing S as well. Hoping that avidity from a tethered client peptide might be helpful, we designed WT::NR (also called DnaK₆₀₉::NR) to present the NRLLLTG peptide into the substrate binding site as in the isolated SBD_U structure (PDBid 1dkz). The hydrolysis-impaired variant T199A DnaK₆₀₉::NR crystallized readily; however, resulting cubic crystals diffracted poorly ($d_{\min} \approx 8\text{\AA}$) and proved intractable at the time. While attempting varied SBD-NR linkers, we solved a structure giving the clue that S-state SBD helix αB may be prone to mid-helix flexibility (Wang *et al.*, 2021), which ultimately led us to construct T199A DnaK₅₄₀::NR. We believe that its high-resolution structure ($d_{\min} = 1.82\text{\AA}$; Table 1) truly represents the predicted S-state. In light of biochemical characterizations of a particular disulfide-bridged construct (Kityk *et al.* 2012), we surmised that it might also be S-like. This proved true for T199A R167C/A480C DnaK₅₄₀::NR ($d_{\min} = 1.92\text{\AA}$; Table 1) after crystallization with a disulfide-bridging ethyl group (Fig. S5A).

With the DnaK₅₄₀ core structure in hand, the cubic structure of T199A DnaK₆₀₉::NR was solved as four copies of this core unit. Only one α -lid domain (residues 537–603, $\alpha B2$ - αE) could be modeled (Fig. S5B), but the lattice is quite open (69% solvent), with ample space for flexibly associated α -lid domains (Fig. S5C). Refinement as quasi-rigid bodies was satisfactory ($R/R_{\text{free}} = 30.4/31.6\%$). Conformational equivalence among DnaK₅₄₀::NR, R167C/A480C DnaK₅₄₀::NR and DnaK₆₀₉::NR structures gives compelling evidence for a stably defined S-state structure with biochemically relevant properties.

Consistent with the allosteric model of Fig. 2A and the excellence of fittings to the hydrolysis data with $K_D^{\text{OR}} = \infty$ (Fig. 2B), the reported DnaK-ATP models would not be expected to bind peptides; however, there are issues. The SBD domain of Qi *et al.* (2013) is compromised by a loop deletion and that of Kityk *et al.* (2012) shows unbuildable SBD outer-loop density for two subunits and quite poor density for the other two. We overcame the problem with T199A E47C/F529C DnaK₆₀₀, which crystallized in a different lattice ($d_{\min} = 2.79\text{\AA}$; Table 1), which has all four SBD β copies well defined (Fig. S5D). Its R-state SBD is unambiguously incompatible with peptide binding, consistent with rapid ATP-induced peptide release. We also analyzed crystals of E47C/F529C DnaK₆₀₉ ($d_{\min} = 2.64\text{\AA}$; Table 1), obtaining the same lattice as for the DnaK₆₀₅::His₆ construct of Kityk *et al.* (2012).

Conformational distinctions and commonalities in the S and R states

The S state of DnaK-ATP is distinguished from its R counterpart by dramatic conformational rearrangements within SBD, structurally modest but functionally critical changes within NBD, and redistribution of SBD relative to NBD. S-state details are rendered at high-resolution in the DnaK₅₄₀::NR and R167C/A480C DnaK₅₄₀::NR structures. The cubic structure of nearly full-length DnaK₆₀₉::NR comprises this same core, but its α -lid extensions adjoin flexibly. The striking distinction of S from R is evident when their similar NBDs are superimposed (Fig. 3A). Whereas SBD α stretches across NBD in R (Fig. 3B of disulfide-bridged DnaK₆₀₀), it extends in reverse from the remodeled and rotated SBD β in S (Fig. 3C of DnaK₆₀₉::NR subunit A with its ordered α -lid domain). Fig. S6 labels the interdomain contact sites in both the S and R states.

In S as in R, SBD β associates with NBD through its NBD-proximal loops and the β -sandwich/barrel axes are roughly aligned (41.8° deviation, Fig. 3D; 18.9° after inner loop superposition, Fig. 3E); however, the two differ radically in mode of association. A rotation of 55.3° about superimposed axes is needed to match SBD β _R to SBD β _S. SBD α _S follows from SBD α _R after a 157.9° rotation of the α A segment and a 60.9° kink for the α A- α B junction, whereby the two α -domains become oppositely directed (Fig. 3D). The two β -domains also differ substantially, with client peptide NR bound to SBD β _S and peptide-binding incompatibility for SBD β _R (Fig. 3E); yet, the NR peptide complex with SBD β _S is essentially identical to that with SBD β _U from the isolated domain (PDBid 1dkz) (Fig. S7A).

Where SBD_S and SBD_U differ markedly is in α -lid dispositions – absent in the DnaK₅₄₀::NR structures and flexibly displaced in DnaK₆₀₉::NR as discussed below. The similarities of SBD_S to SBD_U and of NBD_S to NBD_R are as expected from biochemical characteristics; however, our earlier supposition that the NBD-to-SBD hinge might reside specifically at residue D393 proves to be wrong. Instead, conformational changes are distributed through residues 391–393 at the C-terminus of the linker (Fig. 3F). Moreover, NBD_S is not strictly the same as NBD_R; sub-domain re-orientation angles relating IIA-to-IA, IIB-to-IIA and IB-to-IA are 7.2°, 4.3° and 5.7°, respectively, and they range from 3.2 – 8.5° after superposition based on ATP (Fig. S7B).

The S-state conformation has SBD-NBD contacts at three foci: the NBD-SBD linker junction, SBD loop $\mathcal{L}_{6,7}$ with NBD loop β 10- α F (₁₄₃PAYFND₁₄₈), and the SBD β 8- α A linker (₅₀₅SGLNED₅₁₀) with NBD β 14 and the C-terminus of α K (Fig. 3G). Each of these differentiates S from R: the NBD-SBD linker junctions (Fig. 3F) define the respective orientations of SBD β relative to NBD (Fig. 3D); NBD contacts from both SBD loop $\mathcal{L}_{6,7}$ and the β 8- α A linker impact the site of ATP hydrolysis (Fig. 4 described below); and, reciprocally, SBD contacts from NBD at the β 8- α A linker predispose helix α B to kinkability that affects peptide-binding kinetics (Fig. 5 described below).

The basic similarity of NBD-ATP in S and R states leads to a further commonality, manifest in similar dimeric self-associations for all of the DnaK-ATP structures. Our initial DnaK_S-ATP structure (DnaK₅₄₀::NR) and its R167C/A480C DnaK₅₄₀::NR isomorph both form

nearly the same face-to-face NBD dimer contacts as those found by Qi *et al.* (2013) in $\Delta\mathcal{L}_{3,4}$ DnaK₆₁₀R-ATP (Fig. 3A); our R-state E47C/F529C DnaK₆₀₀ structure comprises similar dimers (Fig. S7C); and, on inspection, DnaK_R-ATP in the original E47C/F529C DnaK₆₀₅::His₆ lattice (Kityk *et al.*, 2012) and in our E47C/F529C DnaK₆₀₉ isomorph the disulfide-bridged structures in the original R-state lattice (PBBids 4b9q & 7ko2) are also similarly dimeric. The dimer interfaces are appreciably plastic, however; quasi-diad axes incline as much as 12° relative to one another (Table S3). The cubic DnaK₆₀₉::NR structure comprises tetramers made up of related, but asymmetric face-to-face dimers (Figs. S8 below). DnaK-ATP is predominantly monomeric in solution; nonetheless, tests of the DnaK_R dimer interface showed its importance for interaction with Hsp40 DnaJ (Sar Beng *et al.*, 2015) and a bioinformatic analysis showed co-variation at dimer contacts (Malinverni *et al.*, 2015). Human Hsp70 also forms dimers, especially when post-translationally modified, and such dimers are present in complexes with Hsp40 (Wu *et al.*, 2020) and with Hsp90 (Morgner *et al.*, 2015). Moreover, the nucleotide-exchange association of Hsp70 with Hsp110 is made through the same kind of face-to-face NBD contacts (Polier *et al.*, 2008; Schuermann *et al.*, 2008; Hendrickson & Liu, 2008).

A second consequence of NBD similarity is commonality in the mode of ATP coordination, which is almost identical in the S and R states, with exception for the P γ phosphate group (Fig. 4A). The nucleotide interacts with all four NBD subdomains (Fig. S7D), and key residues for the reaction mechanism are also distributed among subdomains: K70 (IB), T199 (IIA) and E171 (IA).

Structural basis for regulation of Hsp70 hydrolytic activity

The chaperone activity of Hsp70s depends on the binding and hydrolysis of ATP; but, to be physiologically fruitful, ATP hydrolysis should happen only after the binding of a client substrate. How is ATP hydrolysis regulated to assure this coordination? Our allosteric theory was devised to address the issue, but this then begs the mechanistic question of how Hsp70s restrict hydrolysis in the R-state and release restraints in the S-state. Comparison of the R- and S-state structures of DnaK provides answers when studied in light of the reaction mechanism deduced from our structural analyses on NBD-ATP complexes (Wang & Hendrickson, unpublished; PDBid 7rax).

Earlier structural studies of NBD-mediated ATP hydrolysis used an NBD construct of bovine Hsc70 (Wilbanks *et al.*, 1994) lacking the NBD-SBD linker now known to be critical. We were able to study ATP hydrolysis at high resolution in crystals of ATP complexed with Linker-NBD construct DnaK₃₉₃, using mutant variants and metal replacements to control the rate of hydrolysis (Wang & Hendrickson, PDBid 7rax). These NBD-ATP structures prove to have the S conformation of NBD; and, through the analysis of some 20 NBD crystal structures, we developed a picture of the hydrolytic reaction as it proceeds through a succession of sub-states, starting from a ground state (S_G) as in DnaK₅₄₀::NR and first moving to a primed state (S_P). In S_P, critical water molecule W4 enters between the glycine loop, ₁₉₆GGGT₁₉₉, and the P γ phosphate (Fig. 4A), reorienting P γ for nucleophilic attack. The R state precludes priming for reaction because required movement of the glycine loop is blocked.

With this mechanistic picture in mind, we can understand how the R conformation blocks hydrolysis. R-state restraints in NBD are concentrated in a conformationally distinct segment, $_{220}\text{ATNGDTHLGGED}_{231}$ within $\beta 14$ -to- αH at the IIA-IIB sub-domain boundary, which we call the R-to-S switch segment (Fig. 4B). The switch segment has multiple influences on hydrolysis: (1) The largest switch-segment displacements are within $_{224}\text{DTH}_{226}$, where R-state contacts of carbonyl 225 with the glycine loop ($_{196}\text{GGGT}_{199}$) preclude T199 (IIA) from accommodating the susceptible $\text{P}\gamma$ orientation (Fig. 4B). (2) Switch-segment D231(IIB) salt-bridge hydrogen bonds with R71 (IB) when in the R state, and this draws residue K70 and the associated $\text{P}\gamma$ phosphate away from hydrolytic positioning (Fig. 4C). This restraint also entails hydrogen bonding of H226 (IIA) with D85 (IB) (not shown). Altogether, these contacts are facilitated by the slight subdomain rotations brought about by the lodging of extended SBD helix $\alpha\text{A/B}$ into the IA-IB junction of NBD_R (Fig. 3B). (3) The hydroxyl group of S-state Y145 stabilizes the $\text{P}\gamma$ phosphate via water molecule W1; alternatively, its phenyl group is π - π stacked against the 222–223 peptide bond in $\beta 14$ of R, drawing Y145 away from active-site participation (Fig. 4C).

How R-state restraints check Hsp70 ATPase activity is evident from the R versus S comparison, and the energetics are clear from equilibria between the states. The structure of $\text{NBD}_{393}\text{-ATP}$ at high resolution ($d_{\text{min}} = 1.41\text{\AA}$) clearly has its R-to-S switch segment and catalytic center in the S-state conformation (PDBid 7rax), where the reaction can proceed. The R-S equilibrium for intact Hsp70 strongly favors R (e.g., peptide-free DnaK is only 1.5% S at 20°C), where contacts from SBD serve to block ATP hydrolysis. The active S state becomes increasingly populated in the presence of peptide clients, depending on binding affinity (Hendrickson, 2020), whereby client substrates can be captured for productive chaperone action.

Structural basis for effects of ATP on client peptide binding

ATP strongly affects the binding of client peptides by Hsp70s (Palleros *et al.*, 1993; Schmid *et al.*, 1994; Theyssen *et al.*, 1996; Slepnev & Witt, 1998). Peptide binding is much tighter to Hsp70-ADP (or nucleotide-free Apo) than to Hsp70-ATP, and peptide release is orders of magnitude more rapid from Hsp70-ATP than from Hsp70-ADP/Apo. ATP-induced peptide release is also faster than ATP hydrolysis. Moreover, the on/off kinetics for peptide binding are much faster in ATP than in ADP/Apo. Characteristics of the DnaK_S and DnaK_R structures reported here provide satisfying explanations for these properties.

The $\text{SBD}\beta$ conformation and NR-peptide contacts are nearly identical in the S-state structures as in NR complexes with isolated SBD domains. $\text{SBD}\alpha$ subdomains and their dispositions relative to $\text{SBD}\beta$ do differ, but there are no $\text{SBD}\alpha$ -to-NR contacts; thus, the $\text{SBD}\alpha$ distinctions could only affect binding thermodynamics indirectly. Yet, because peptide binding requires $\text{SBD}\alpha$ movement, we expect these distinctions to impact binding kinetics. The αB kinking found in the vicinity of DnaK 536–538 in an SBD_U -NR polymorphs (PDBid 1dky) and in a variant NR fusion protein (PDBid 7kzi) point to the required accommodation.

The structure of $\text{DnaK}_{609}::\text{NR}$ embodies the very mobility suggested above. Although the construct purified as a monomer, self-associated to the tethered NR peptide (Fig. S1), it

crystallized as a C2-symmetric tetramer of screw-displaced face-to-face dimers (Fig. S8); however, only the NBD-SBD β - α A- α B1 core of each subunit is well ordered. Places needed for the α -lid domains (α B2- α C- α D- α E) are at voids in the ordered lattice of core domains (Fig. S5C). One α -lid domains could be modeled into difference densities, but the simplest general explanation is that all S-state α -lid domains are rigid-body extensions from a disjointed α B helix; the ordered one adventitiously engages packing contacts (Fig. S5C). Such α -lid mobility is consistent with the rapid peptide-binding kinetics in ATP. After ATP hydrolysis, SBD_U uncouples from NBD_U, which frees SBD α to form the hydrogen-bonded latch with SBD β that restricts binding-site access.

Why is the α -lid domain so much more flexibly associated for DnaK-peptide complexes in ATP than when in ADP? The answer is found in the high-resolution S-state structure of DnaK₅₄₀::NR, where SBD interacts intimately with NBD through its β 8- α A linker (Fig. 3G). These contacts displace α A in SBD_S relative to isolated SBD_U, and this change in turn affects the disposition of α B relative to α A and SBD β (Fig. 5A). Stabilizing contacts between α B1 and inner SBD β loops remain intact, but we found when building α -lid domains into the DnaK₆₀₀::NR structure that extending α B straight along the α B1 trajectory generates collisions with SBD β loops (Fig. 5B). Thus, S-state constraints preclude α -lid latch interactions and, given the aforementioned intrinsic plasticity in the middle of α B, highly varied α -lid orientations arise (Figs. 5C&D). With the peptide-binding site uncovered in Hsp70_S, inevitably the on/off rates for peptide binding are elevated relative to Hsp70_U.

Upon ATP hydrolysis, SBD_S-associated client peptides are captured in tight SBD_U complexes and retained so after ADP dissociates. The NR peptide binds identically to SBD β _S as to SBD β _U, making five backbone-to-backbone hydrogen bonds between NRLLLTG and loops $\mathcal{L}_{1,2}$ and $\mathcal{L}_{3,4}$ and burying the central leucyl side chain into a pocket lined by hydrophobes I401, F426, V436 and I438 (Figs. 5E & S9A). Peptide release is very rapid when ATP rebinds, a hallmark of Hsp70 allostery. The previous R-state structures (PDBids 4b9q & 4jne) show opened peptide-binding sites, and our E47C/F529C DnaK₆₀₀ structure provides a definitive model for SBD_R. Substrate peptides cannot remain bound to SBD β _R, where $\mathcal{L}_{3,4}$ is prised apart from $\mathcal{L}_{2,3}$ (Fig. 3E) and F426 directly contacts I438 and eliminates the leucyl pocket (Figs. 5F & S9B).

What accounts for this peptide-ejecting ATP-induced conformational change in SBD β _R? The answer lies in the NBD-SBD interface of Hsp70_R, which provides a stereospecific link between the ATP and peptide-binding sites (Fig. 5G). This interface has two parts – NBD contacts with SBD inner loops $\mathcal{L}_{L,1}$, $\mathcal{L}_{2,3}$, and $\mathcal{L}_{6,7}$ appear to act as a fulcrum against which contacts forming with α A/B and $\mathcal{L}_{4,5}$ serve to draw strand β 8 into associations with both β -sheets and to flatten the binding site (Wang & Hendrickson, 2021). The less extensive interface of Hsp70_S (Fig. 5H) imparts no distortive pressure on ground-state SBD β , defined by 389SBD_U-NR (PDBid 1dkz); however, due to β 8- α A linker contacts noted above, there is a direct linkage between ATP and the consequently labile α -lid conjunction in Hsp70_S (Fig. 5I).

Characterization of mutant variants at S-state interfaces

We seek insights into allosteric regulation of Hsp70 activity by mutational analysis of domain interfaces. Given the allosteric equilibrium between R and S states, a mutant variant designed to disrupt a functional interface in one state might be expected to tip the equilibrium toward the other state. Alternatively, an interface mutant might actually stabilize its state. Ideally, a candidate mutation would show functional exclusivity, that is, it should affect properties of one state without significantly impacting the other. As deduced from its constitutive S-state phenotype (Fig. 1D–G), I483D is such a mutation: I483 is buried into the SBD-NBD interface of DnaK_R-ATP, but it is exposed innocuously on the surface of DnaK_S-ATP. Other sites are less propitious for exclusivity.

Relevant interfaces in DnaK_S-ATP involve NBD contacts with SBD loop $\mathcal{L}_{6,7}$ and the SBD α -SBD β junction (Fig. 3G); however, several of the same residues also participate in DnaK_R-ATP interfaces, some are also engaged in intra-domain contacts, and others interact only through backbone hydrogen bonding. This complicates mutational analysis, but biochemical assays can ascertain the equilibrated balance. For example, D481 has a central place in the R-state interface, but it also makes crucial S-state contacts (Figs. S9C&D); and R167 and A480 are proximate in both R and S. Nevertheless, as for I483D, D481A and K mutants show constitutive S-like biochemical properties (Kityk *et al.*, 2015); and disulfide-bridged R167C-A480C is S-like in biochemistry (Kityk *et al.*, 2012) and structure (Table 1). All but one NBD contact with the S-state SBD α -SBD β junction is by backbone-to-backbone hydrogen bonding, and thus not directly mutation sensitive. The carboxylate groups of D148 and D479 make another key contact, but this too is not completely state exclusive and uncomplicated; D148 also hydrogen bonds into a backbone R-state contact, and D479 also makes an intra-SBD hydrogen bond in both states.

While recognizing possible ambiguities for interpretation, we proceeded to test strategic mutations at positions 148 and 479. We also tested replacements for E217 that were inspired by our earlier hypothetical S-state model (Liu & Hendrickson, 2013); although actually only nearby to an authentic S-state interface, E217R had shown R-like biochemical properties (Fig. S10A). Cell viability tests of several variants at 148, 479 and 217 showed severely impaired chaperone activity, as did selected double and triple mutants (Figs. 6B & S10B). We next tested four of the most defective mutants (D148V, D148I and D148I/D479S/E217R combinations) in the state-sensitive fluorescence assay. For each, we found the fluorescence quenched and blue-shifted to beyond wild-type levels, consistent with imposition of the R state. Amazingly, for these D148 mutants unlike WT DnaK or any previously analyzed mutant, W102 quenching prevails for ADP as well as ATP, although not when nucleotide-free (Figs. 6B & S10C).

Hydrophobic replacements for D148 do not merely disrupt the S state; rather, they actively stabilize an R-like conformation. So much so, that the R-state contacts of SBD α A/B with NBD are in place even with ADP, evident because the spectral shifts are due to hydrophobic contacts from SBD α L507 and M515 to NBD W102 that modulate indole transitions (Liu & Hendrickson, 2007; Qi *et al.*, 2013). Indeed, modeling places the isoleucine of D148I into an R-interface pocket where it contacts hydrophobic side chains of L454, I483 and L484 (Figs.

6C&D), further stabilizing the intrinsically favored R state. By contrast, the R-state interface is highly sensitive to I483 mutation; I483D, L, T, H and R all have negligible chaperone activity (Fig. S3A). I483 is nestled against hydrophobic portions of NBD residues in the R state (Fig. S9C), where aspartic acid is not tolerated; but it is located clear of interfaces in the S alternative (Fig. S9D). We conclude that the S-state interface seen in our structures is essential for Hsp70 chaperone activity.

I483D and D148I are complementary, state-specifying mutations at identically conserved sites on the uncoupled Hsp70_U surface. Both mutants destroy chaperone activity, the former by exclusive promotion of the stimulating S-state alternative and the latter by exclusive stabilization of the restraining R-state alternative (Fig. 6B).

DISCUSSION

Hsp70-mediated chaperone activity involves cyclic iterations of the binding and release of client polypeptides coordinated with the binding and hydrolysis of ATP. It is through the allosteric interplay of these activities, as ATP is consumed, that entangled polypeptides might be disaggregated and promoted to a native conformation. The cycle of Hsp70 activity was first depicted biochemically by Palleros *et al.* (1993) and then structurally by Zhu *et al.* (1996). Other versions have followed, and now we can complete the Hsp70 chaperone cycle with insights from the R-S equilibrium and in full atomic detail (Fig. 7).

Each step of the Hsp70 cycle is represented in Fig. 7A by an experimental DnaK structure. Entering the cycle at upper left, we find the uncoupled ADP state Hsp70_U (ADP, client) having ADP bound to NBD and a client polypeptide captured in SBD. ADP is released as we proceed clockwise to yield Hsp70_U (Apo, client) (upper right), perhaps with the assistance of a nucleotide exchange factor such GrpE (bacteria) or Hsp110 (metazoans). Restraining state Hsp70_R (ATP, client-free) forms as ATP binds and the client polypeptide is ejected (lower right). Hsp70_R can equilibrate to the stimulating state Hsp70_S (lower left), which unlike Hsp70_R can accept a client peptide, recycled or new, thereby generating Hsp70_S (ATP, rebound client). Productive ATP hydrolysis now yields Hsp70_U (ADP, client) and reinitiates the chaperone cycle. Iterations of the cycle disentangle misfolded or aggregated proteins (Sharma *et al.*, 2010; Imamoglu *et al.*, 2020) and release them for Anfinsen (1973) refolding to the native state.

It is the conformational equilibrium between S and R, established as ATP binds to U(Apo), that fosters allosteric coupling between the ATP and peptide binding sites; however, it is the direct connections across the NBD-SBD interfaces and through subdomains NBD IA and SBD β that actually mediate coupling between the two active sites (Figs. 5G–I). The R-state interface perturbs SBD β such that the outer loops are flattened and peptide binding is precluded; whereas the S-state interface stabilizes SBD β in a conformation that readmits peptide binding between loops $\mathcal{L}_{1,2}$ and $\mathcal{L}_{3,4}$ as in SBD_U, but which imposes a mid- α B flexure that loosens the α -lid subdomain (Fig. 7B). The presence of a suitable client peptide drives the equilibria toward SP, away from R, and enhances hydrolysis to UP(ADP). Thereby, the client polypeptide is captured and isolated until ATP rebinding expels it – as a refolded protein if favored.

Limitations of Study

Our allosteric theory accounts for much of Hsp70 biochemistry, but it is simple for tractability; whereas, reality is likely more complex. We provide biochemical evidence for similar peptide-free (S) and complexed (SP) properties; still, a structure for S is desired to complement SP. This quest was frustrated, likely by loop dynamics that limit crystallization feasibility. Quite generally, crystallography is limited in its depiction of dynamics, and this is most apparent here for α -lid conformations. On the other hand, structures described here provide excellent starting points for computational simulations of ATP hydrolysis, peptide binding, and R-to-S-to-U transitions.

STAR*Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for reagents should be directed to the lead contact, Wayne A. Hendrickson wah2@cumc.columbia.edu.

Materials Availability—All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

Data and Code Availability

- *Data*: Atomic coordinates and structure factors for the five crystal structures have been deposited in the Protein Data Bank under accession codes 7krt, 7ko2, 7kru, 7krv, and 7krw as designated in Table 1.
- *Code*: This paper does not report original code.
- *Other*: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial cells—This paper does not report original code.

E. coli cell cultures were used for protein production and chaperone activity assays. Plasmids were transformed into RosettaTM (DE3) cells (Novagen) for protein expression and for cell viability assays. Mutagenesis experiments were performed in *E. coli* 10G cells (Lucigen).

METHOD DETAILS

Plasmids, strains and protein expression—A *dnaK* expression plasmid pBB46 (*amp^R*), which produces full-length DnaK (Burkholder *et al.* 1996), was modified to add a C-terminal six-histidine affinity-purification tag. Site-directed mutants were constructed using QuikChange Mutagenesis (Stratagene, La Jolla, CA). Addition of the His₆ tag had no obvious effect on the growth phenotype for either WT or mutant DnaK (data not shown). All DnaK proteins were expressed in a *dnaK* deletion strain BB205 (*cam^R kan^R*, Burkholder *et al.*, 1996) to eliminate native WT DnaK protein contamination since Hsp70 self-associates during purification. Induction was done at 30°C with 1mM IPTG as the final concentration

for 5 hours in LB medium supplemented with 50 µg/ml ampicillin, 25 µg/ml kanamycin, and 25 µg/ml chloramphenicol.

The WT::NR fusion construct was amplified by PCR with the 3' primer including the NR peptide sequence fused to DnaK (residues 1–609) through a flexible linker (sequence: GGSGGSGS). The PCR product was cloned into the pSMT3 vector (Mossessova & Lima, 2000), a gift from Dr. Chris Lima, through BamHI and XhoI sites. After transforming into *E. coli* expression strain BL21(DE3) Gold, expression was done at 30°C with 1mM IPTG as the final concentration.

Protein purification—To purify the WT and point-mutant DnaK proteins, cells were pelleted after induction by centrifugation at 5,000 g and resuspended in ice-cold 2X-PBS (20 mM Na₂HPO₄, 1.76mM KH₂PO₄, 274mM NaCl and 5.4mM KCl). All purification steps were performed at 4°C. Before loading onto a HisTrap column (GE Healthcare), sonication was done in ice-water bath for 3 minutes followed by centrifugation at 12,000 g. DnaK proteins eluted from the HisTrap column were first dialyzed again 2X-PBS overnight, further purified with a HiTrap Q column (GE Healthcare), and evaluated by PAGE (Fig. S1A). Fractions containing DnaK protein were pooled, concentrated to >20mg/ml in a buffer containing 10 mM Hepes-KOH, pH 7.5, and 50 mM KCl, and flash frozen in liquid nitrogen.

The WT::NR fusion protein was first purified using a HisTrap column as described above. After dialysis against 2X-PBS overnight, the Smt3 tag was cleaved off by Ulp1 protease, and removed by loading onto a HisTrap column. The WT::NR fusion protein was further purified with a HiTrap Q column as described above, and a Superdex 200 column (26/60), saving the monomeric fraction (Fig. S1B). After concentration to >20mg/ml, samples were flash frozen in liquid nitrogen.

Tryptophan fluorescence assay—Emission spectra from the single tryptophan residue in DnaK proteins were measured on a FluoroMax-3 fluorescence spectrometer (Horiba Scientific). Excitation was set at 290 nm and emission spectra were collected from 300 to 400 nm. DnaK proteins at final concentration of 1µM were first incubated with either 2mM ATP or ADP for two minutes in buffer A (25 mM Hepes-KOH, pH 7.5, 100 mM KCl, and 10 mM Mg(OAc)₂), and spectra were then recorded at ~25°C. The peak reading for each DnaK protein in ADP was set at 1.0, and the relative fluorescence intensity for both ATP and ADP spectra were plotted versus wavelength (Fig. S2B–H).

Single-turnover ATPase assay—The assay was performed as described previously (Davis *et al.*, 1999) with several modifications. For DnaK-ATP complex formation, DnaK (10 µg) was incubated with ~50 µCi of [α -³²P]ATP (Perkin Elmer) in 100 µl buffer A containing 2 µM ATP for 5 minutes on ice. The DnaK-ATP complex was isolated immediately from free ATP on a spin column (GE Healthcare), and flash frozen with liquid nitrogen. Single-turnover ATPase assays were performed at 20°C in buffer A containing various concentrations of peptide NR (NRLLLTG). The maximum solubility of NR is ~1.5 mM, which limits the accessible range. At the specified times, the reaction was stopped, and ATP was separated from ADP using thin-layer chromatography (TLC). The

percent conversion of ATP to ADP was determined after quantification using a Typhoon phosphor-imaging system (GE Healthcare). For each single-turnover reaction, we took nine time points (Figs. S4A&B). The rate of ATP hydrolysis (k_{cat}) was determined by fitting to a first-order rate equation by nonlinear regression analysis using PRISM (GraphPad, San Diego, CA).

Fluorescence anisotropy assay of peptide-binding—Peptide NR (NRLLLTG) was labeled at the N-terminus with fluorescein (F-NR, synthesized by Sigma Aldrich) for use in fluorescence anisotropy assays as previously described (Liu *et al.*, 2001). Serial dilutions of DnaK proteins were prepared in buffer A with either 100 μM ADP or 2 mM ATP, and then incubated with 10 nM F-NR at room temperature to allow binding to reach equilibrium. Fluorescence polarization was measured on a QuantaMaster1 fluorescence spectrometer (Photon Technology International, South Brunswick, NJ) with excitation at 490 nm and emission at 535 nm. To calculate K_d , fluorescence anisotropy data were fitted to a one-site binding equation by nonlinear regression analysis using PRISM (GraphPad, San Diego, CA).

To test F-NR release upon ATP binding, 10 μM DnaK protein was incubated with 10 nM F-NR at room temperature in the presence of 100 μM ADP in buffer A for several hours to reach equilibrium. At this concentration of DnaK, more than 80% F-NR was bound to DnaK protein at equilibrium. ATP was added to final concentration of 2 mM, and fluorescence anisotropy was measured continuously at five second intervals (Fig. S4E).

Cell viability tests—Growth tests were performed in *E. coli* as described previously (Liu & Hendrickson, 2007) with modifications. DnaK pBB46 plasmids (*amp^R*) carrying mutant *dnak* were transformed into a *dnak* deletion strain BB205 (*cam^Rkan^R*). Since *E. coli* growth requires a functional DnaK at 37°C and above, but not at 30°C, growth tests were carried out at elevated temperatures with control tests at 30°C. For each mutant, a fresh overnight culture was prepared from a fresh transformation. 5 μl serial dilutions of the overnight cultures were spotted on LB plates containing 50 $\mu\text{g/ml}$ ampicillin, 25 $\mu\text{g/ml}$ kanamycin, 25 $\mu\text{g/ml}$ chloramphenicol, and 20 μM IPTG, and grown one overnight at 37°C to 40°C to evaluate viability.

Fitting to conformational equilibrium theory—We developed a least-squares program for the fitting of parameters to our theoretical model of peptide-stimulated ATP hydrolysis by Hsp70 proteins. The observed k_{cat} values are weighted by their inverse variances, $w = 1/\sigma^2$, where $\sigma = (\sigma_{\text{fit}}^2 + \sigma_{\text{sys}}^2)^{1/2}$ where σ_{fit} is the random error deduced from fitting to the kinetic data (e.g. Fig. S4A) and σ_{sys} is a systematic error increment to account for added variations in repeated constant measurements, as for N170D, V389D and I483D at varying peptide concentrations (Table S1). We find $\sigma_{\text{sys}} = 0.0324 \times k_{\text{cat}}$.

Crystallization—Peak SEC fractions for the specified DnaK protein were pooled, concentrated to approximately 10 mg/ml, and set up for crystallization by the vapor diffusion technique in 96-well plates (Art Robbins 102-0001-20) using a Mosquito nanoliter pipetting robot (TTP labtech), both for initial screening and for further optimization. Typically, 900 nl of protein solution was added to 300 nl from a precipitant matrix, either

the JCSG suite (Qiagen), JCSG+ and PACT (Molecular Dimensions), or Crystal Screen HT and Index HT (Hampton Research). Crystallization trays were placed at two different temperatures, 4°C and 20°C in a Rock Imager (Formulatrix). Plates were examined every day for the first week, and then every few days for the rest of the first month following a Fibonacci sequence.

Once an initial condition was found, crystallizations were then tested in optimization trials. First, a grid optimization of pH and main precipitant concentration gradients was tested to find the appropriate composition of these two parameters for best diffraction. Second, additives to the mother liquor identified by grid optimization were tested at a ratio of 1:8. The additives used included JCSG I-IV (Qiagen), and Silver Bullets and Additives HT (Hampton Research). Ultimate crystallization conditions differ for the various crystal structures in this study as specified below in relation to identifications in Table 1:

All crystallizations and structural analyses here used T199A variants of the indicated proteins.

DnaK₅₄₀::NR. Protein solution: T199A DnaK₅₄₀::NR, 2 mM ATP, 5 mM MgCl₂ in Buffer A. Precipitant solution: 0.04 M HEPES pH 7.5, 25.5% PEG4000, 15% glycerol, 0.17 M ammonium sulfate, 0.1M KCl. Temperature: 4°C. In order for the correct conformation to form, the proteins were directly applied to crystallizations after size exclusion chromatography, without buffer exchange or a centrifugal concentrating process. The protein solution was mixed with well solution at volume ratio 3:1 or higher, in order to reach supersaturation.

R167C/A480C DnaK₅₄₀::NR. Protein solution: T199A/R167C/A480C DnaK₅₄₀::NR after reaction with M2M (1,2-ethanediyl bismethanethiosulfonate, Loo and Clarke, 2001) to produce the ethyl-bridged disulfide bond, 2 mM ATP, 5 mM MgCl₂ in Buffer A. Precipitant solution: 0.04 M HEPES pH 7.5, 25.5% PEG4000, 15% glycerol, 0.17 M ammonium sulfate, 0.01 M citric acid, 3% PEG 6000. Temperature: 4°C.

Although the R167C/A480C mutant protein behaved well, as for Kityk *et al.* (2012), it did not crystallize as oxidized in air or by copper phenanthroline. MTS cross linkers M3M and M4M were also tried, but only the reaction product from M2M crystallized. We presume that the alternatives were distortive.

DnaK₆₀₉::NR. Protein solution: T199A DnaK₆₀₉::NR, 2 mM ATP, 5 mM MgCl₂ in Buffer A. Precipitant solution: 0.1 M HEPES pH 7, 3.2 M ammonium sulfate. Temperature: 4°C.

E47C/F529C DnaK₆₀₉. Protein solution: T199A/E47C/F529C DnaK₆₀₉, 2 mM ATP, 5 mM MgCl₂ in Buffer A. Precipitant solution: 0.1 M sodium cacodylate pH 6.5, 30% PEG 8000, 0.2 M sodium acetate. Temperature: 4°C. The protein product from the Smt3/Ulp1 procedure used here terminates exactly at residue 609, whereas the His₆-tag remained in the procedure used by Kityk *et al.* (2012); crystallization conditions differed somewhat different as well, using 28% PEG 4000 and pH 5.6 at 18°C. Nevertheless, the crystal lattice obtained here is isostructural with the predecessor.

E47C/F529C DnaK₆₀₀:: Protein solution: T199A/E47C/F529C DnaK₆₀₀, 2 mM ATP, 5 mM MgCl₂ in Buffer A. Precipitant solution: 0.2 M magnesium acetate, 0.1 M sodium cacodylate pH 6.5, 20% PEG 8000. Temperature: 4°C.

Diffraction measurements—Crystals were mounted on LithoLoops (Molecular Dimensions), cryoprotected with 20%–25% glycerol or LV CryoOil (MiTeGen), plunge-frozen into liquid nitrogen, and stored in ALS-style pucks (MiTeGen). Pucks were then loaded into a canister in a dry shipper, and sent to the Northeastern Collaborative Access Team (NE-CAT) at the Advanced Photon Source (APS) of Argonne National Laboratory (ANL). NE-CAT beamline 24-ID-E has x-ray energy fixed at 12.66 keV, and the variable energy of 24-ID-C was also set at 12.66 keV ($\lambda = 0.97918 \text{ \AA}$). Both beam lines are equipped with state-of-the-art photon detectors with noiseless and ultra-fast readout capability. All data sets were collected in N₂ gas streams boiled off from liquid nitrogen and held at 100 K. Measurements were controlled through the remote collection interface from NE-CAT.

After data collection, data sets were transferred from NE-CAT server to local disks. Diffraction data were first integrated and scaled by XDS (Kabsch, 2010), then merged and truncated by AIMLESS (Evans & Murshudov, 2013). Data processing was straightforward from one single crystal except for two cases:

DnaK₆₀₉::NR. Data processing for the cubic lattice was complicated by intergrown lattices. These data were collected in a vector ϕ -scan of a 10- μ beam across a single large (~400 μ) crystal in 0.2° steps through 360°. The data processing revealed that this ‘crystal’ was in fact a mosaic comprising several separable lattices. Accordingly, the data were processed in wedges. We separated up to seven lattices per 10°-wedge and integrated reflections with XDS-based outlier rejection of overlaps. A final data set with 26.7-fold multiplicity was compiled and merged from 146 wedged components.

E47C/F529C DnaK₆₀₀:: These crystals were relatively small and the lattice symmetry (monoclinic) is low. Accordingly, data were merged from three crystals to achieve adequate multiplicity and precision.

Structure determination—Structures were determined by molecular replacement with Phaser (McCoy et al., 2007) and refined with PHENIX (Adams *et al.*, 2010).

DnaK₅₄₀::NR. Molecular replacement searches were made with NBD from the DnaK-ATP structure of Qi *et al.* (2013, PDBid 4jne) and with SBD β of Zhu *et al.* (1996, PDBid 1dkz), residues 393–502. After rigid-body refinements of two copies each, SBD α portions were built into difference densities and the structure was refined with appropriate stereochemical restraints.

R167C/A480C DnaK₅₄₀::NR. The crystal lattice of this structure is isomorphous with that of DnaK₅₄₀::NR, which was used as the starting point for refinement after building of the ethane-bridged disulfide unit.

DnaK₆₀₉::NR. The analysis of this cubic structure was complicated by having multiple copies in the asymmetric unit (4–6 from solvent content considerations) and by limited diffraction ($d_{\min} = 7.7\text{\AA}$). Subunit B from the structure of DnaK₅₄₀::NR was used for various molecular replacement searches. An initial solution having five copies in the asymmetric unit failed to refine well, which led us to alternative searches focused on $Z_a = 4$. We found few solutions with the packing function of Phaser enabled, and the top solution did in fact have collisions. Ultimately, the eighth-rated solution with the packing function disabled was collision-free and refined satisfactorily as four rigid bodies. The presence of twinning (twin law: $h, k, l \rightarrow -l, -k, -h$) was revealed by evaluation with phenix.xtriage, and the twin fraction ultimately refined to 44%.

The structure was first refined with rigid-body refinement, with the core of each chain (residues 2–536) defined as a rigid-body group. This was followed by five rounds of quasi-rigid-body refinement, where the four units were tied tightly to the reference model and to Ramachandran restraints, to NCS equivalence with torsion-based NCS restraints, and to the diffraction data by fixing wxc_scale at 0.2 for tight control of geometry terms. Each group was given a single isotropic atomic displacement parameter (adp or B-factor). This refinement yielded satisfactory R-values ($R_{\text{work}}/R_{\text{free}} = 0.321/0.329$).

At this point, a composite omit map was searched for residual density to be associated with α -lid subdomains in interstitial spaces in the lattice (Fig. S5B). Density was found for two copies. One appeared to comprise multiple conformers that were unable to model; however, a single conformer could be fitted to the other. Continued quasi-rigid-body refinement, now including the α -lid domain of chain A (residues 537–603) as a fifth body. Its inclusion reduced the R-values appreciably on refinement ($R_{\text{work}}/R_{\text{free}} = 0.304/0.316$) while preserving the excellent reference geometry.

Although non-crystallographic symmetry was not sought in the searches, a two-fold symmetric association of two screw-diad-related face-to-face dimers obtains in the tetrameric structure (Fig. S8, Table S3), providing added validation for the solution.

E47C/F529C DnaK₆₀₉. The crystal lattice of this structure is isomorphous with that of the DnaK-ATP structure of Kityk *et al.* (2012, PDBid 4b9q), which was used as the starting point for the refinement.

E47C/F529C DnaK₆₀₀. Molecular replacement searches were made with the model of a subunit from the original lattice of E47C/F529C DnaK₆₀₉. In order to achieve unbiased images of R-state SBD β , outer-loop segments were deleted to calculate omit maps that were then fitted *de novo* and refined.

Structural analysis narrative—Distinctive biochemical characteristics expected in DnaK-ATP_S allowed us to design suitable S-state constructs. We first crystallized T199A DnaK₆₀₉::NR, a hydrolysis-impaired variant of WT::NR; however, resulting cubic crystals diffracted poorly ($d_{\min} \approx 8\text{\AA}$) and, having several copies per asymmetric unit, they initially proved intractable. We then built a hypothetical model and also tested other constructs. Ultimately, we succeeded in obtaining high-resolution S-state structures from truncated

constructs, and we were then able to solve the four-copy cubic structure. We also designed a variant construct for an R-state structure that gave a well-resolved structure of the opened, peptide-free SBD β conformation.

The rate of ATP hydrolysis is elevated similarly in NBD constructs that delete SBD but retain the linker as for peptide-stimulated WT DnaK (Vogel *et al.*, 2006; Swain *et al.*, 2007). Thus, we expected the NBD-linker unit of DnaK-ATP_S to be structurally similar to that of DnaK-ATP_R (Qi *et al.*, 2013). Because the K_{DS} for binding of various peptides to various binding-site mutants (Mayer *et al.*, 2000) correlate well for DnaK in ATP versus ADP and because binding-site geometries for the two cases are similar as observed in EPR studies of labeled peptides (Popp *et al.*, 2005), we expected the SBD structure in DnaK-ATP_S to be similar to that in isolated SBD-peptide complexes (Zhu *et al.*, 1996) and in DnaK-ADP-peptide complexes (Bertelsen *et al.*, 2009). Nevertheless, the DnaK-ATP_S structure must have communicative NBD(ATP)-SBD contacts since peptide-binding kinetics are affected strongly by the presence of ATP (Schmid *et al.*, 1994; Table S2). Accordingly, we built a structural model of DnaK-ATP_S by adjusting only the Ramachandran angles at D393 while seeking compatible NBD-SBD interactions. We obtained a plausible model with SBD re-oriented by 134° from DnaK-ATP_R (Liu & Hendrickson, 2013).

The WT::NR construct was designed, based on the isolated SBD structure (Zhu *et al.*, 1996), to present the NR peptide in the substrate binding site. Also called DnaK₆₀₉::NR, it has DnaK2–609 fused through a flexible -GGSGSG- linker to the NRLLLTG heptapeptide. Because the resulting crystals diffracted poorly, we wondered if this linker might be non-optimal and tried several variations. One alternative led to a crystal structure with some expected S-state properties, but with the NBD-SBD linker partially disengaged and with a kink in the long α B helix (Wang & Hendrickson, 2021). This kink is reminiscent of an 11° kink between residues 536 and 538 of α B in type I SBD, breaking it into α B1 and α B2 in the type II SBD structure (Zhu *et al.*, 1996), and its displaced α -lid domain (residues 538–607) conjured up an explanation for elevated on/off peptide-binding kinetics in ATP as compared to ADP (Schmid *et al.*, 1994). Being also aware of truncated bovine Hsc70 (Jiang *et al.*, 2005) and *C. elegans* Hsp70 SBD structures (PDBid: 2op6 & 3dob) that have α B peptides in the binding site, we then produced truncated NR fusion protein DnaK₅₄₀::NR, introducing a -TTGSG- linker between D540 of DnaK and the NR heptapeptide.

Initial crystallizations of DnaK₅₄₀::NR generated a lattice-swapped state (Wang & Hendrickson, 2021), which inspired our Q-intermediate hypothesis but did not satisfy expectations for the S state. Realizing that interdomain associations must have come undone in the crystallization media, we adjusted conditions to assure monomeric integrity. Thereupon, we obtained a high-resolution structure ($d_{\min} = 1.82\text{\AA}$; Table 1) that we believe truly represents the predicted S-state. We also noted that characterizations by Kityk *et al.* (2012) of a disulfide-bridged construct designed to stabilize their Sse1-based homology model of DnaK, now defined as the R-state, actually has biochemical properties like those of the S-state (Fig. 2A). Accordingly, we produced R167C/A480C DnaK₅₄₀::NR. Its crystallization required disulfide linkage mediated by an MTS-introduced ethyl group, presumably because the direct disulfide bond is somewhat distortive. Excellent crystals grew

($d_{\min} = 1.92\text{\AA}$; Table 1) with this ethyl bridging in place (Fig. S5A), and the resulting structure is essentially identical to S-state DnaK₅₄₀::NR itself.

With these putative S-state structures in hand, we returned to the cubic crystals of DnaK₆₀₉::NR. Diffraction data were improved from new crystals, but with the resolution limit only marginally better ($d_{\min} = 7.7\text{\AA}$; Table 1). Nevertheless, molecular-replacement searches with the DnaK₅₄₀::NR model now produced a solution with four copies of this core structure. The resulting lattice is spare (69% solvent) with ample interstitial spaces to accommodate the α -lid domains (Fig. S5C). A ($F_O - F_C$) Fourier-difference map showed densities for two lid domains (residues 537–603, $\alpha B2 - \alpha E$), one of which could be modeled fully occupied (Fig. S5B). Refinement as quasi-rigid bodies was satisfactory ($R/R_{\text{free}} = 30.4/31.6\%$). Conformational equivalence among DnaK₅₄₀::NR, R167C/A480C DnaK₅₄₀::NR and DnaK₆₀₉::NR structures provides compelling evidence for a stably defined S-state structure with biochemically relevant properties.

The allosteric model embodied in Fig. 2A and the excellence of fittings to the hydrolysis data (Fig. 2B) with $K_D^{\text{OR}} = \infty$ imply that R-state SBD cannot bind client peptides. The reported DnaK-ATP models support this conclusion. Yet, the SBD domain of Qi *et al.* (2013) is compromised by a loop deletion and that of Kityk *et al.* (2012) shows unbuildable SBD outer-loop density for two subunits and quite poor density for the other two. Noting clashes of C-termini from lattice mates with these SBD tips, we generated and crystallized E47C/F529C DnaK₆₀₀, which shortens helix αE and deletes the His₆ tag from E47C/F529C DnaK₆₀₅::His₆. Although overall resolution is not improved in a new lattice ($d_{\min} = 2.79\text{\AA}$; Table 1), peptide-binding loop definitions are now definitive (Fig. S4C) in all four copies of the new lattice. Thus, the R-state conformation of SBD is unambiguously incompatible with peptide binding, consistent with the rapid peptide release seen on ATP binding (Schmid *et al.*, 1994). We also analyzed crystals of E47C/F529C DnaK₆₀₉ ($d_{\min} = 2.64\text{\AA}$; Table 1) and obtained the same lattice as that for E47C/F529C DnaK₆₀₅::His₆.

Structure comparisons and modeling—The atomic model for the uncoupled U state as complexed with ADP was revised from the initial model of Bertelsen *et al.* (2009), for which the NBD component had been adjusted from the NBD in a complex of apo DnaK with GrpE (PDBId 1dkg; Harrison *et al.*, 1997) to fit RDC data. We revised this model by replacing that NBD with our DnaK₃₈₆-ADP structure (PDBId: 7n46; Wang & Hendrickson, unpublished). We also replaced the SBD component, which had mistakenly used coordinates from the selenomethionyl version (PDBId 1dkx), with the isostructural natural version of SBD (PDBId 1dkz; Zhu *et al.*, 1996).

PyMOL (Schrödinger) was used in building the model for the D148I mutant structure, using the PyMOL mutagenesis function; for superpositions in structure comparisons (described here using conventions from TOSS (Hendrickson, 1979); and in preparing structure figures.

QUANTIFICATION AND STATISTICAL ANALYSIS

Rates of ATP hydrolysis (k_{cat}) were determined by fitting of kinetic data with a first-order rate equation by nonlinear regression analysis using PRISM (GraphPad, San Diego, CA). Fitting errors (σ_{fit}) were augmented in quadrature with a systematic error component (σ_{sys})

ascertained by adjustment to match actual variations in repeated measurements of hydrolysis by proteins (N170D, V389D and I483D) showing rates essentially invariant with peptide concentration (Table S2). Fittings obtained by Mathematica (Wolfram Research) was used to fit Eq. 3 to k_{cat} measurements as a function of [NR]; and, assuming the experimental value for K_D^{OS} , the other biochemical parameters (k' , k^0 and K_{Eqs}) were evaluated from the mathematical parameters (a, b and d) with errors propagated standardly. Peptide binding constants (K_d values of Table S2) were fitted to fluorescence anisotropy data with one-site binding equation by nonlinear regression analysis using PRISM (GraphPad, San Diego, CA). Diffraction measurements were reduced to integrated, scaled and merged structure factors by XDS (Kabsch, 2010) and AIMLESS (Evans & Murshudov, 2013). Structure refinements were performed with PHENIX (Adams *et al.*, 2010) and structure validations were performed with MOLPROBITY (Chen *et al.*, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Construction and biochemical characterization of mutant variant DnaK proteins.

(A) Schematics of Hsp70 domain structure. Boundaries and sites of mutation in *E. coli* DnaK are marked (upper) and the WT::NR fusion protein is shown (lower). Color assignments: NBD blue, NBD-SBD Linker purple, SBD β green, SBD α red and NR peptide (NRLLLTG) orange.

(B) Ribbon diagram of DnaK-ATP_R showing mutated residues. Backbone coloring is as in **1A**; side chains of mutated residues and W102 are in orange and yellow, respectively.

(C) Diagrammatic illustration of the WT::NR fusion protein. Coloring is as in **1A**.

(D) Allosteric coupling. The ADP-to-ATP shift in wavelength of maximal fluorescence for each variant is compared to that for WT DnaK.

(E) Effect of substrate peptide binding on ATP hydrolysis. ATP hydrolysis as measured by single-turnover kinetics is compared to WT for each variant minus peptide substrates and plus 400 μ M NR peptide.

(F) Peptide binding in the presence of ADP (100 μ M). Fluorescence anisotropy measured from fluorescein-labeled NR peptide (10 nM) as a function of DnaK concentration for each variant in comparison to WT DnaK. The key associates symbols with proteins.

(G) Peptide binding in the presence of ATP (2 mM). Binding was assayed as in **1F**, but in ATP instead of ADP with symbols as in **1F**.

Figure 2. Theory for allosteric control of ATP hydrolysis by Hsp70 proteins.

(A) Theoretical model. The restraining state of Hsp70_R-ATP (**R**) is postulated here to have no affinity for substrate peptide and to hydrolyze ATP at the basal rate k' to Hsp70_U-ADP (**U**). Hsp70_R-ATP (**R**) is proposed to be in equilibrium (K_{eqS}) with stimulating state Hsp70_S-ATP (**S**), which in the presence of substrate peptide (P_S) is in equilibrium (K_D^{OS}) with the complex Hsp70_S-ATP-peptide (**SP**). Both **S** and **SP** hydrolyze ATP at an elevated rate k' to **U** and **UP**, respectively. Structural cartoons are color coded in accord with 1A. Orange dots (●) connote peptide substrates and (◂) symbols connote α-lid mobility.

(B) Fittings of the allosteric theory to hydrolysis data. ATP hydrolysis rates measured by single-turnover kinetics at 20°C (Table S1), are shown as a function of NR peptide concentration [P], for WT DnaK and for the I483D and WT::NR mutant variants. The fitting

to the WT data by theory Eq. (3) is drawn in purple, and the fitted asymptotic value $k'(\text{WT})$ is shown as a horizontal line in comparison to the observed measurements for the fully disengaged I483D and WT::NR proteins where $K_{\text{eqS}} = 0$ and $k_{\text{cat}} = k'$. The effective local concentration on NR peptide in the WT::NR fusion protein is estimated to be 6.8 mM based on the length of flexible linker (Zhou, 2001).

Figure 3. Conformational distinctions in the S and R states.

(A) Comparison of R- and S-state structures. R ($\Delta\mathcal{L}_{3,4}$ DnaK₆₁₀) and S (DnaK₅₄₀::NR) dimers are drawn as ribbons with the NBDs of right-side protomers superimposed. The right-side S is colored by domain as in 1A; otherwise, R is pink and S is light blue with left-side partners subdued.

(B) R-state structure of E47C/F529C DnaK₆₀₀. The polypeptide trace of chain D is shown in worm representation with coloring as in 1A.

(C) S-state structure of DnaK₆₀₉::NR (chain A). NBD is oriented as superimposed onto that of R in 3B. Presentation is as in 3B with the NR peptide orange.

(D) Comparison of SBD domains with NBD domains (surface rendering) superimposed. Ribbons are shown from 387 onward. Coloring: S (light blue), S axis blue), R (pink), R axis (red), NR (orange).

(E) Comparison of SBD β subdomains as superimposed on inner loops. Coloring as in **3D**.

(F) Comparison of NBD-SBD linker segments. Coloring as in **3D**.

(G) S-state interface of SBD β (green) and SBD α (red) with NBD (blue). Contacts of SBD loop $\mathcal{L}_{6,7}$ (479–485) with NBD loop $\beta_{10-\alpha F}$ (143–151) and of the SBD $\beta_{8-\alpha A}$ linker (505–510) with NBD β_{14} (219–222) of the R-S switch segment and the end of αK (326–328), as well as an ATP interaction, are shown in atomic detail. Selected NBD segments are shown as backbone worms.

Figure 4. Structural basis for regulation of ATP hydrolysis.

(A) Relative dispositions of groups associated with the P γ phosphate group of ATP as bound in conformational states S (blue), R (pink) & S_p (olive).

(B) Comparison of R-S switch segments (220–231) and associated glycine loops (196–199) and ATP in S (blue) and R (pink) states.

(C) Atomic details of R-S switch segment associations with the glycine loop (196–199) and NBD α C (70, 71) and of tyrosine Y145 in S (blue) and R (pink) interactions.

Figure 5. Structural basis for effects of ATP on peptide-binding kinetics.

(A) SBD α disposition in DnaK_S-ATP (green and red) compared with that in the isolated domain, SBD_U (grey). Superposition is based on SBD β .

(B) Collisions (*Molprobit* clash symbols in magenta) that would occur between residues of helix α B and outer loops $L_{3,4}$ and $L_{5,6}$ if α B were extended as in SBD_U from residue 536 of SBD_S in 5A.

(C & D) Orthogonal views of DnaK₆₀₉::NR. The experimental α -lid domain of subunit A from the cubic-lattice tetramer (Fig. S8) is in red, and four alternatively disposed α -lid models that fit into lattice voids are subdued. (A) SBD orientation similar to 5A but with α A vertical. (B) View rotated 90° to the left of 5A.

(E) SBD β surface in the S-state complex of DnaK₅₄₀::NR. The NR peptide is an orange worm with stick bonds for central leucine residue L4. The SBD β surface is grey except for L4 pocket-lining residues V436, I438, F426 and I472 (cyan).

(F) SBD β surface in the R-state structure of DnaK₆₀₀. The rendering is as for **5E**, except for the L4 pocket-lining residues (pink). R is superimposed on S based on inner loop segments 319–412 and 443–452, and peptide NR is included from S for reference.

(G-I) Allosteric interaction pathways between ATP and client peptide sites. ATP is drawn as stick bonds and relevant protein segments are drawn as C α backbones in **1A** domain coloring. Each panel has the same orientation and scaling. **(G)** Pathway from ATP to peptide-contacting loops $\mathcal{L}_{1,2}$ and $\mathcal{L}_{3,4}$ in R-state DnaK. **(H)** Disrupted ATP-peptide pathway in S-state DnaK. **(I)** Pathway from ATP to α -lid displacements in S-state DnaK.

Figure 6. Characterization of mutant variants at the S-state interface.

(A) Cell viability tests of chaperone activity for mutations at E217, D148 and D479.

Expression plasmids mutated as indicated were introduced in appropriate null backgrounds and tested in serial dilutions for overnight growth on agar plates at 40° C for heat stress.

(B) Tryptophan fluorescence assays of DnaK conformation for indicated mutant proteins, testing each in ADP and in ATP. Wavelengths of peak fluorescence are indicated by vertical bars.

(C) R-state structure in the vicinity of D148, showing associated water molecules and H-bonding from the carboxylate group to backbone NH 484. Domain coloring has NBD (blue), SBD β (green), and NBD-SBD linker (purple).

(D) R-state structure modeled for the D148I mutant, drawn as for **6C** but replacing waters and showing vdW contacts of the mutant isoleucine with hydrophobic side chains.

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Figure 7. Hsp70 chaperone cycle.

(A) The cycle in DnaK atomic structures. Proceeding clockwise from **upper left**, DnaK_U(ADP, peptide) is first in an uncoupled state with ADP/Pi bound to NBD and a client polypeptide bound to SBD; DnaK_U (apo, peptide) obtains after release of ADP (**upper right**); rebinding of ATP to NBD generates DnaK_R (ATP, no client) with consequent release of the polypeptide (**lower right**); the equilibrium between R and S then permits the rebinding of a client polypeptide to SBD in the S state, DnaK_S (ATP, rebound client); and ATP hydrolysis breaks the NBD-SBD coupling and returns to the starting point (**lower left**). Each step of the cycle is represented here by an experiment-based structure: DnaK_U (ADP) is adapted from the NMR-based model for the disposition of SBD relative to NBD (PDBid 2kho), but has NBD replaced by our actual structure of the ADP complex (PDBid 7n46);

DnaK_U (apo) has NBD replaced by the empty domain from the complex with GrpE (PDBid 1dkg); and DnaK_R and DnaK_U are from this work. Coloring is as in **1A** except that SBD α A is yellow to distinguish it in the R state.

(B) Schematic drawings of peptide sites apo in Hsp70_U (right), Hsp70_R (center), and Hsp70_S (left). Segments from the NBD-SB linker through β 8 are drawn faithfully within schematic SBD β outlines viewed from the side. Domain outlines are colored as in **1A**, with NBD lobe I a lighter blue and lobe II darker. As in **2A**, orange dots (●) connote bound peptides and (◀) symbols connote α -lid mobility. Orientations are roughly from the right of corresponding panels in **7A** panels. The S-state distance from ATP P γ to C α of the NR central leucine is 46 Å.

Table 1.

Diffraction data and refinement

	DnaK ₅₄₀ ::NR	R167C/A480C DnaK ₅₄₀ ::NR	DnaK ₆₀₉ ::NR	E47C/F529C DnaK ₆₀₀	E47C/F529C DnaK ₆₀₉
Data collection					
Beamline	NE-CAT 24-ID-C	NE-CAT 24-ID-E	NE-CAT 24-ID-E	NE-CAT 24-ID-E	NE-CAT 24-ID-C
Wavelength (Å)	0.97918	0.97918	0.97918	0.97918	0.97918
Bragg spacings (Å)	46.21–1.82 (1.89–1.82)	49.35–1.92 (1.99–1.92)	37.36–7.71 (7.98–7.71)	47.02–2.79 (2.89–2.79)	47.75–2.64 (2.73–2.64)
Space group	P6 ₅ 22	P6 ₅ 22	I23	P2 ₁	C2
Unit cell dimensions					
a, b, c (Å)	98.52 98.52 384.75	98.70 98.70 382.61	294.20 294.20 294.20	77.47 199.53 94.23	202.43 77.49 182.74
α, β, γ (°)	90 90 120	90 90 120	90 90 90	90 93.7 90	90 101.8 90
Za / solvent content (%)	2 / 46.2	2 / 45.4	4 / 69.0	4 / 56.0	4 / 53.7
Total reflections	3256327 (317537)	941932 (89194)	136474 (13111)	1006546 (96063)	2284183 (231999)
Unique reflections	100002 (9767)	84697 (8157)	5107 (510)	70764 (7064)	81891 (8136)
Multiplicity	32.6 (32.5)	11.1 (10.9)	26.7 (25.5)	14.2 (13.6)	27.9 (28.5)
Completeness (%)	99.9 (99.7)	99.6 (97.2)	97.1 (99.2)	99.6 (99.3)	99.8 (99.4)
Mean I/sigma(I)	13.47 (0.62)	8.59 (0.60)	8.97 (0.56)	5.74 (0.39)	9.88 (0.69)
R _{merge}	0.259 (4.71)	0.200 (2.50)	0.632 (3.70)	0.713 (3.25)	0.554 (2.42)
R _{meas}	0.263 (4.78)	0.210 (2.62)	0.644 (3.77)	0.739 (3.38)	0.565 (2.46)
R _{pim}	0.046 (0.83)	0.062 (0.78)	0.121 (0.74)	0.195 (0.91)	0.108 (0.46)
CC _{1/2}	0.999 (0.35)	0.998 (0.36)	0.947 (0.41)	0.784 (0.28)	0.938 (0.57)
CC*	1.000 (0.72)	0.999 (0.73)	0.986 (0.76)	0.938 (0.66)	0.984 (0.85)
Refinement					
Reflections used for R _{work}	98148 (9558)	82615 (7896)	4757 (483)	69150 (6878)	79726 (7888)
Reflections used for R _{free}	1812 (177)	1999 (191)	258 (27)	1535 (146)	2043 (201)
R _{work}	0.178 (0.294)	0.195 (0.331)	0.304 (0.429)	0.279 (0.409)	0.241 (0.325)
R _{free}	0.209 (0.321)	0.239 (0.368)	0.316 (0.528)	0.308 (0.425)	0.287 (0.362)
Number of non-hydrogen atoms					
macromolecules	9140	9004	17030	17919	18470
ligands	8270	8262	16906	17752	18082
solvent	125	112	124	128	133
Protein residues	745	630	0	39	255
RMSD ideality (bonds, Å)	1079	1079	2231	2414	2399
RMSD ideality (angles, °)	0.007	0.012	0.009	0.004	0.004
Ramachandran favored (%)	0.83	1.15	1.37	0.70	0.69
Ramachandran allowed (%)	99.3	99.1	98.8	99.0	99.0
Ramachandran allowed (%)	0.8	0.9	1.0	1.0	1.0
Rama-Z (whole structure)	0.74	0.04	0.66	2.34	1.92
Rotamer outliers (%)	0.3	0.9	1.0	0.5	0.5

	DnaK₅₄₀::NR	R167C/A480C DnaK₅₄₀::NR	DnaK₆₀₉::NR	E47C/F529C DnaK₆₀₀	E47C/F529C DnaK₆₀₉
Clashscore	1.43	3.16	16.01	2.96	4.20
Average B-factor (Å ²)	39.6	41.5	320.0	61.7	74.8
macromolecules	39.0	41.3	320.0	61.8	75.3
ligands	53.7	46.6	320.0	50.4	51.2
solvent	43.8	42.5	N/A	41.1	47.2
PDB ID	7KRU	7KRV	7KRW	7KRT	7KO2

All of these DnaK constructs include the hydrolysis-impairing T199A mutation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Rosetta™ (DE3) cells	Novagen	Cat#70954
E. cloni® 10G cells	Lucigen	Cat#60107
BB205 <i>E. coli</i> cells	Burkholder et al., 1996	N/A
Chemicals, peptides, and recombinant proteins		
Oligopeptide NRLLLTG	Sigma Aldrich	N/A
DnaK ₅₄₀ ::NR	This paper	N/A
R167C/A480C DnaK ₅₄₀ ::NR	This paper	N/A
DnaK ₆₀₉ ::NR	This paper	N/A
E47C/F529C DnaK ₆₀₀ ::NR	This paper	N/A
Isopropyl-β-D-thiogalactoside (IPTG)	CHEM IMPEX	Cat#00194
LB Broth, Ready-Made Powder	Affymetrix	Cat#75852
Ampicillin, sodium salt USP	Bio Basic	Cat#AB0028
Kanamycin USP Grade	Goldbio	Cat#K-120-25
Chloramphenicol	Bio Basic	Cat#CB0118-50
Adenosine 5-triphosphate disodium salt hydrate 99%	Sigma-Aldrich	Cat#A26209
Adenosine 5-diphosphate monopotassium salt dihydrate 95%	Sigma-Aldrich	Cat#A5285
1,2-ethanediyl bismethanethiosulfonate	Santa Cruz Biotechnology	Cat#sc-208746
Dichloro(1,10-phenanthroline)copper(II)	TCI Chemicals	Cat#D3891-5G
LV CryoOil	MiTeGen	Cat#LVCO-5
HisTrap HP	GE Healthcare	Cat#17-5248-02
HiTrap® Q High Performance	GE Healthcare	Cat#17-1154-01
Superdex™ 200 Increase 10/300 GL Prepacked	GE Healthcare	Cat#28990944
Critical commercial assays		
Expressplus™ PAGE Gel	GenScript	Cat#M42015
NeXtal Tubes JCSG Core I suite	Qiagen	Cat#130724
NeXtal Tubes JCSG Core II suite	Qiagen	Cat#130725
NeXtal Tubes JCSG Core III suite	Qiagen	Cat#130726
NeXtal Tubes JCSG Core IV suite	Qiagen	Cat#130727
JCSG-plus suite	Molecular Dimensions	Cat#MD1-37
PACT premier	Molecular Dimensions	Cat#MD1-29
Crystal Screen HT	Hampton Research	Cat#HR2-130
Index HT	Hampton Research	Cat#HR2-134
Silver Bullets	Hampton Research	Cat#HR2-096
Additive Screen HT	Hampton Research	Cat#HR2-138
Deposited data		
Crystal structure of DnaK ₅₄₀ ::NR	This paper	PDBid 7kru

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Crystal structure of R167C/A480C DnaK ₅₄₀ ::NR	This paper	PDBid 7krv
Crystal structure of DnaK ₆₀₉ ::NR	This paper	PDBid 7krw
Crystal structure of DnaK ₆₀₉ ::NR	This paper	PDBid 7krw
Crystal structure of E47C/F529C DnaK ₆₀₀ ::NR	This paper	PDBid 7krt
Crystal structure of E47C/F529C DnaK ₆₀₉ ::NR	This paper	PDBid 7ko2
Recombinant DNA		
pSMT3	Mossessova & Lima, 2000	N/A
pBB46	Burkholder et al., 1996	N/A
Plasmids based on pSMT3 for expression of the recombinant proteins used for crystallization in Table 1 (see METHOD DETAILS)	This paper	N/A
Plasmids based on pBB46 for expression of the recombinant proteins used for cell viability tests (see METHOD DETAILS)	This paper	N/A
Software and algorithms		
XDS	Kabsch, 2010	https://xds.mr.mpg.de/
CCP4	M. D. Winn et al., 2011	https://www.ccp4.ac.uk/
PHENIX	D. Liebschner et al., 2019	https://phenix-online.org/
Wolfram Mathematica	Wolfram	https://www.wolfram.com/mathematica/
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
PyMOL	Schrödinger	https://pymol.org/2/
COOT	Emsley et al., 2010	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
Clustal Omega	Sievers and Higgins, 2018	https://www.ebi.ac.uk/Tools/msa/clustalo/
Other		
FluoroMax-3 fluorescence spectrometer	Horiba Scientific	N/A
Typhoon phosphor-imaging system	GE Healthcare	N/A
QuantaMaster1 fluorescence spectrometer	Photon Technology International	N/A
96-well plates	Art Robbins	Cat#102-0001-20
Rock Imager	Formulatrix	N/A
Mosquito nanoliter pipetting robot	TTP labtech	N/A