Supporting Information

Structural Basis for the Inhibition of HSP70 and DnaK Chaperones by Small-Molecule Targeting of a C-Terminal Allosteric Pocket

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TABLE OF CONTENTS

Figure S1. PET-16 binds directly to the nucleotide-free HSP70 protein.

Table S1. Data collection and refinement statistics for the DnaK-PET-16 structure.

Figure S2. X-ray crystal structure of the DnaK-PET-16 complex.

Figure S3. The DnaK-PET-16 complex is structurally distinct from the ADP- and ATP-bound DnaK structures in the absence of PET-16.

Results and Discussion
The PET-16-binding site has different conformations in unliganded DnaK structures and in substrate-bound structures.

Methods
Reagents, antibodies, mass spectrometry, electron microscopy, plasmids, proteins, protein crystallization, structure determination, isothermal titration calorimetry studies, and soaking PES and PET-16 into existing crystals.

References

Table S2. Data collection and refinement statistics for the unliganded DnaK structures.

Table S3. Summary of comparative analyses.

Figure S4. PET-16 binding modulates the DnaK structure relative to the unliganded form.

Table S4. Data collection and refinement statistics for the substrate-bound DnaK structure.

Figure S5. PET-16 binding modulates the DnaK structure relative to the substrate-bound protein.
**Figure S1.** PET-16 binds directly to the nucleotide-free HSP70 protein. Representative ITC assays of PET-16 with purified full-length, nucleotide-free HSP70 protein. The data shown are representative of two independent experiments. The reported dissociation constant is the average and standard deviation from two independent experiments.
### Table S1. Data Collection and Refinement Statistics for the DnaK-PET-16 Structure

#### Data collection

| Space group          | P4\(_3\)2\(_1\)2 |
|----------------------|------------------|
| **Cell dimensions**  |                  |
| \(a\), \(b\), \(c\) (Å) | 91.78, 91.78, 136.89 |
| \(\alpha\), \(\beta\), \(\gamma\) (°) | 90.00, 90.00, 90.00 |
| Wavelength           | 1.100            |
| Resolution (Å)       | 45-3.45 (3.57-3.45) |
| \(R_{merge}\)        | 9.9 (49.0)       |
| \(I/\sigma(I)\)      | 15.0 (2.5)       |
| Data completeness (%)| 99.7 (99.9)      |
| Redundancy           | 5.0 (5.3)        |
| No. reflections      | 8281             |

#### Refinement statistics

| Resolution (Å)       | 45-3.45 (3.57-3.45) |
| No. reflections      | 8148               |
| \(R_{work}/R_{free}\) | 28.43/32.82        |
| No. atoms            | 2831               |
| Protein              | 2803               |
| Ligand/ion           | 27                 |
| Water                | 1                  |
| B-factor (Å\(^2\))   |                    |
| Average              | 43.46              |
| Protein              | 43.37              |
| PET-16               | 54.52              |
| r.m.s deviations     |                    |
| Bond lengths (Å)     | 0.0045             |
| Bond angles (°)      | 1.081              |

r.m.s., root-mean-square deviation. Values in parentheses are for the highest resolution shell.
**Figure S2.** X-ray crystal structure of the DnaK-PET-16 complex. (A) Stereo view of Figure 3B. The $2F_o-F_c$ electron density map of the refined structure corresponding to PET-16 contoured at 1.0 σ is shown in blue. The $F_o-F_c$ difference map prior to introducing PET-16 into the model is contoured at 3.0 σ and shown in green, there is no contribution from PET-16 in this map. $F_o-F_c$ PET-16 omit map contoured at 3.0 σ is shown in red. Side chain of key PET-16 contacting
residues and Gly482 are shown in stick format and labeled. The PET-16 compound is shown as a stick model in gray. Note that PET-16 binds to a pocket formed by strand β1 (L399) and loops LL,1 (L392, P396), L6,7 (G482) and Lα,β (A503 and S504). (B and C) 90° rotated views of the image shown in A. (D) An expanded view of the PET-16 binding pocket. A 2F_o-F_c electron density map contoured at 1.0 σ of the refined structure is shown (orange for DnaK and blue for PET-16) with the protein shown in blue lines and PET-16 shown in gray sticks. (E) Same view as in Figure 3B except that F_o-F_c PET-16 omit map contoured at 1 σ (green), 3 σ (red), and 5 σ (blue).
Figure S3. The DnaK-PET-16 complex is structurally distinct from the ADP- and ATP-bound DnaK structures in the absence of PET-16. (A) Top panel, structural comparisons of the DnaK-PET-16 complex with the ADP-bound DnaK. The PET-16 compound is shown in red. Bottom panel, structural differences noted in loops LL,1 and Lα,β of the DnaK-PET-16 and ADP-bound DnaK structures are presented. The PET-16 compound is shown in cyan. (B and C) Structural comparisons of the DnaK-PET-16 complex with the ATP-bound DnaK X-ray structures. (D-F) A surface representation of the corresponding PET-16 cleft (as shown in Figure 4A) in the ADP-DnaK (D) and ATP-DnaK (E and F) structures. The PET-16 compound is shown as a stick model in blue. Note that the ADP-bound DnaK (D) easily accommodates the PET-16
compound. However, for the structures shown in (E and F), the PET-16 binding site is blocked, as indicated by the shadow representation of the PET-16 molecule.
RESULTS AND DISCUSSION

The PET-16-Binding Site has Different Conformations in Unliganded DnaK Structures. To assess the status of the PET-16 binding pocket in the unliganded DnaK state, we overlaid structurally corresponding Cα atoms of the SBD of DnaK-PET-16 with the following reported structures: DnaK-SBDβ NMR structure (residues 393-507, PDB code 1DG4)\(^1\) and DnaK-SBD X-ray structure (residues 389-607, PDB code 4F01)\(^2\). We also generated three independent unliganded DnaK-SBD X-ray structures (referred to here as DnaK-SBD-A, DnaK-SBD-B and DnaK-SBD-C), which were refined to 2.36 Å, 1.75 Å and 2.97 Å (Supplementary Table S2). These structures were compared to the DnaK-PET-16 structure. As shown, the overall DnaK-PET-16 structure superimposes well with various DnaK structures (DnaK-SBD, DnaK-SBD-A, DnaK-SBD-B and DnaK-SBD-C all in Supplementary Table S3). However, the interaction between PET-16 and the C-terminus of DnaK brings residue L392 in loop LL,1 and residue S504 in Lα,β closer together by ~ 5.5-8.1 Å relative to the corresponding residues in the unliganded structures (Supplementary Figures S4A and S4B). This observation provides additional evidence that when PET-16 interacts with residues such as L392 and S504, there is an alteration of the conformation of loops LL,1 and Lα,β (Figure 3E; Supplementary Figures S3A, S4A and S4B).

The DnaK-PET-16 structure also displays several conformational differences relative to the reported unliganded DnaK-SBDβ, particularly in the loops of SBDβ and along strands β3, β7 and β8 (Supplementary Figure S4C). Notably, in the surface diagrams, the PET-16-binding cavity is not available in the DnaK-SBDβ structure (Supplementary Figure S4C). Indeed, we found by ITC analyses that the interaction between PET-16 and DnaK (aa 393-507) was reduced by approximately seven-fold, with a Kd value of ~ 41 µM; this compares to a Kd value of ~ 5.9 µM with DnaK (aa 389-607). Correspondingly, we obtained co-crystals of PET-16 with DnaK (aa 389-607), but not with DnaK (aa 393-507), consistent with the idea that the latter
structure does not provide the appropriate surface for PET-16 binding.

The PET-16-Binding Site has Different Conformations in Substrate-Bound Structures. We next compared the DnaK-PET-16 structure to the reported DnaK-NRLLLTG X-ray structures (PDB codes 1DKZ and 1DKY) and to a NRLLLTG-liganded DnaK-SBD co-crystal complex that we obtained (referred to here as DnaK-NRLLLTG-A); the latter is refined to 1.98 Å with excellent stereochemistry (Supplementary Table S4). The overall fold of the SBDβ of our DnaK-NRLLLTG-A is virtually identical to the reported DnaK-NRLLLTG structure (PDB code 1DKZ), with a rmsd of 0.28 Å (Supplementary Table S3). However, the SBDβ domains of DnaK-NRLLLTG (PDB code 1DKY) and DnaK-NRLLLTG-A vary from the SBDβ of DnaK-PET-16, with rmsds of 0.732 Å and 0.764 Å, respectively (Supplementary Table S3). In particular, the interactions among PET-16, strand β1 and loops LL,1 and Lα,β bring residues P396 and L399 closer to residue S504 by an average of 3.5 Å and 4.6 Å, respectively (Supplementary Figures S5A and S5B). Also, in the presence of PET-16, the distance between residue M404 in Region B and residue S504 in Region A shortens by an average of ~ 4.6 Å, relative to their positions in the substrate-bound structures (Supplementary Figures S5A and S5B). These observations point to localized structural changes in the protein following substrate binding, which could impact the interaction between PET-16 and HSP70 as well as between PET-16 and DnaK. Thus, we used ITC to ask whether pre-incubation of ADP-bound HSP70 with NRLLLTG might alter the interaction between the protein and PET-16. Indeed, under these conditions, the binding affinity of PET-16 for the HSP70 protein bound to substrate was reduced by approximately seven-fold, with a $K_d$ value of ~ 28.4 μM; this compares to a $K_d$ value of ~ 3.8 μM in the absence of peptide (Figure 2C). These combined findings suggest that PET-16 likely interacts preferentially with the client-free, ADP-bound form of HSP70 and DnaK. In support of this premise, we were unable to generate co-crystals of DnaK-SBD with PET-16 by soaking the
DnaK-NRLLLTG-A crystals with varying concentrations of PET-16.

METHODS

Reagents and Antibodies. The primary and secondary antibodies used in this work were described previously.\textsuperscript{4,5} Sulfo-NHS-SS-Biotin (Biotin) and PES (2-Phenylethynesulfonamide or Pifithrin-\(\mu\)) were purchased from Pierce Biotechnology, Inc. (Rockford, IL) and EMD Millipore Chemicals, Inc. (Billerica, MA), respectively. The HSP70 antibodies used for the affinity purification studies were from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The other antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). As previously detailed\textsuperscript{4} four distinct shRNA sequences against human HSP70 as well as negative controls (hairpin control, sh-Negative) were purchased from OriGene Technologies, Inc. (Rockville, MD), and transfected using the Lipofectamine LTX Reagent with PLUS Reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions.

Mass Spectrometry and Electron Microscopy. Liquid chromatography-tandem mass spectrometry was performed by the Genomics Institute and Abramson Cancer Center Proteomics Core Facility at the Perelman School of Medicine, University of Pennsylvania. Electron microscopy (EM) and EM imaging were performed by The Electron Microscopy Resource Laboratory, Biomedical Research Core Facilities, at the Perelman School of Medicine, University of Pennsylvania.

Plasmids and Proteins. The human stress-inducible HSP70 (residues 1-641 or 386-616) and \textit{E. coli} DnaK (residues 1-638, 389-607, or 393-507) were cloned into the pET25 vector (EMD Millipore Chemicals, Inc., Billerica, MA, USA) between the \textit{NdeI} and \textit{XhoI} restriction sites. The full-length HSP70 and DnaK mutants were constructed by PCR-based site-directed
mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA). All plasmid inserts were verified by DNA sequencing. All HSP70 and DnaK variants were produced with a N-terminal His$_6$-tag in the *E. coli* BL21 Star (DE3) strain (Life Technologies catalog number C6010-03). The resulting strains were grown at 37°C in LB medium containing 50 µg/ml of carbenicillin (Sigma-Aldrich Co., St. Louis, MO, USA). At an OD$_{600}$ ~0.3-0.5, protein expression was induced with 0.5 mM IPTG, and cells were subsequently grown at 25 °C for ~16 h. Cells were collected by centrifugation and resuspended in the Bugbuster Master Mix (EMD Millipore Chemicals catalog number 71456-4), supplemented with protease inhibitors, 1 mM DTT and 20 mM imidazole. The His$_6$-tagged fusion proteins were isolated on Ni$^{2+}$-chelating resins (Ni-NTA Superflow, Qiagen catalog number 30410) by standard procedures. When necessary, size-exclusion chromatography on a Superdex-200 or a Superose 6 10/300 GL (GE Healthcare) analytical column pre-equilibrated with 1X DPBS (Life Technologies catalog number 14190-136), 1 mM Tris-HCl (pH 7.4) or 10 mM Tris-HCl (pH 7) was used as the final purification step. The purified DnaK proteins (residues 389-607 or 393-507) were dialyzed thoroughly against 1X DPBS with 5 mM DTT at 4°C, for crystallization trials involving the small molecule inhibitor PET-16 and the substrate peptide NRLLLTG. For PES cocrystallization studies, the DnaK proteins were dialyzed thoroughly against 1X DPBS. All HSP70 and DnaK variants were dialyzed extensively against the buffers as noted below under “Isothermal Titration Calorimetry Studies” for the ITC analyses. The dialyzed proteins were subsequently microcentrifugated at 15,000 x g for 5 min at 4°C, and aliquots of the soluble proteins were stored at -80°C.

**Protein Crystallization and Structure Determination.** Crystals were prepared by mixing 400 µM of DnaK (residues 389-607 or 393-507) with 4 mM PET-16, 4 mM PES or 1.33 mM NRLLLTG (Biomatik, Wilmington, Delaware, USA), with DMSO added to a 1.6% final
concentration. DMSO was added to fully resuspend PES, because this small molecule has poor solubility in 1X DPBS (Life Technologies catalog number 14190-136). In contrast, PET-16, which is a bromine salt, is soluble to 100 mM in water and 1X DPBS. The protein mixtures were preheated to 42°C for 15 minutes and gradually cooled to room temperature (referred to here as the heat shock method). We chose the heat shock method, because we only obtained co-crystals of DnaK-PET-16 using this approach. In this report, we also generated four additional control crystals using the heat shock method: DnaK-SBD-A, DnaK-SBD-B, DnaK-SBD-C and DnaK-NRLLLTG-A. These unliganded and peptide-bound structures were refined to 2.36 Å, 1.75 Å, 2.97 Å and 1.98 Å, respectively (Supplementary Tables S2 and S4). The protein preparations were subjected to centrifugation at 15,000 x g for 5 min at room temperature, and the soluble protein complexes were subjected to the crystallization screening using the hanging-drop vapor diffusion method. The drops were set up with 1 µl protein plus 1 µl reservoir solution at room temperature; the resulting drops contained 0.8% DMSO. Crystals of DnaK (aa 389-607), alone and in complex with either PET-16 or NRLLLTG, were obtained. Although we obtained crystals of DnaK (aa 389-607) in the presence of PES, the crystals did not contain electron density corresponding to PES. No crystals of DnaK (aa 393-507) in the presence of PET-16, NRLLLTG or PES were obtained. Crystals of DnaK grown in the presence of PET-16 were obtained from a solution of 1.8 M ammonium sulfate and 0.1 M Bis-Tris, pH 5.5 at room temperature. DnaK-NRLLLTG-A crystals grew in a hanging-drop set up at room temperature with 2.2 M ammonium sulfate and 0.1 M Bis-Tris, pH 5.5. Two unliganded DnaK-SBD X-ray structures (DnaK-SBD-A and DnaK-SBD-B) were grown by vapor diffusion at room temperature from a solution containing 0.15 M potassium bromide and 30% w/v polyethylene glycol monomethyl ether 2000. The third unliganded DnaK-SBD-C structure was grown by vapor diffusion at room temperature from a solution containing 2.1 M ammonium sulfate and 0.1 M Bis-Tris, pH 5.5. The DnaK-SBD-B crystals resulted from the DnaK (aa 389-607) protein mixtures that had been incubated with PET-16, but electron density corresponding to PET-16
was not observed in the refined structures. The DnaK-SBD-A structure was obtained by soaking DnaK-SBD-B crystals with PET-16 for 24 h; however, the electron density corresponding to PET-16 was not observed in the refined structure. The DnaK-SBD-C crystals resulted from the DnaK (aa 389-607) protein mixtures that had been incubated with PES, but electron density corresponding to PES was not observed in the refined structure. Crystals were harvested into the reservoir solution with the addition of glycerol (25% v/v) and flash-frozen in liquid nitrogen. All diffraction data sets were collected at the National Synchrotron Light Source (Brookhaven National Laboratory) and processed with HKL2000. The DnaK-PET-16, DnaK-NRLLLTG-A and DnaK-SBD-C data sets were collected on beamline X25, and data sets for DnaK-SBD-A and DnaK-SBD-B were collected on beamline X29A. The crystal structures of all five structures were determined by molecular replacement with PHASER, using the structure of DnaK-NRLLLTG (PDB code 1DKY) as a search model, with the NRLLLTG peptide extracted from the search model. The structures of the proteins were refined and manually adjusted using the high resolution native dataset by iterative cycles of refinement with Phenix.refine, and model building was performed using the program COOT. The PET-16 and NRLLLTG ligands were modeled into the corresponding electron density maps after refinement of the protein had converged. Note, the density assigned to the small molecule inhibitor PET-16 is not an alternative linker conformation; the linker density is well defined in the DnaK-PET-16 structure, even at very low contour. The final model, and the modeled PET-16 inhibitor, was checked for errors with a composite omit map generated by AutoBuild in the Phenix suite. Data collection and refinement statistics are shown in Supplementary Tables S1, S2 and S4. All the structural figures were prepared using PYMOL (http://www.pymol.org/). Note, the observed PET-16 interacting pocket in the DnaK-PET-16 structure is also visible in the DnaK-NRLLLTG structures (PDB codes 1DKZ and 1DKY) and the HSP70-NRLLLTG structure (PDB code 4PO2).
Isothermal Titration Calorimetry Studies. All ITC experiments were performed using a MicroCal VP-ITC isothermal titration calorimeter (MicroCal). The following proteins were prepared in 1 mM Tris-HCl (pH 7.4): full-length HSP70, full-length DnaK, and DnaK (aa 393-507). The HSP70 (aa 386-616) protein was prepared in 10 mM Tris-HCl (pH 7) buffer. Immediately before the ITC run, we added to the purified protein (in 1 mM Tris-HCl, pH 7.4 or 10 mM Tris-HCl, pH 7) an aliquot of DMSO to a final concentration of 3%, along with ATP or ADP, as indicated. Note, we only added ADP or ATP to the protein mixtures. We also prepared the PET-16, PES and NRLLLTG solutions in 1 mM Tris-HCl (pH 7.4) or 10 mM Tris-HCl (pH 7), as indicated, with DMSO added to a 3% final concentration. The 3% DMSO was included to fully resuspend PES, which has low solubility in 1 mM Tris-HCl (pH 7.4) buffer as well as 10 mM Tris-HCl (pH 7) buffer. In contrast, PET-16 as a bromine salt is soluble to 100 mM in water, 1 mM Tris-HCl (pH 7.4) buffer and 10 mM Tris-HCl (pH 7) buffer. Since the goal of the ITC studies was to compare the binding affinity of PES to NRLLLTG and PET-16, we also supplemented all ITC reactions involving PET-16 and NRLLLTG with 3% DMSO. The syringe was loaded with protein mixtures with pH values of approximately 5.5. The calorimeter chamber was loaded with PES, PET-16 or NRLLLTG solutions prepared in the same buffer as the protein mixtures; the inhibitor and peptide solutions also had pH values of approximately 5.5. All solutions were degassed for 5 min. The titrations involving the full-length ADP-bound or ATP-bound proteins were performed at 25°C. The titrations involving the purified HSP70 (aa 386-616), DnaK (aa 393-507) and full-length nucleotide-free proteins were performed at 42°C. For each experiment, we performed 24 injections of 12 µl of protein solution (syringe) into the ~ 1.4 ml calorimeter chamber containing PES, PET-16 or NRLLLTG solution. The stirring speed was set at 310 revolutions per minute and a 300 s delay time was maintained between each injection. At the completion of the ITC run, the resulting inhibitor-protein mixture or the peptide-protein mixture in the ITC chamber had a pH value of approximately 5.5. Interestingly, a significant portion of intracellular HSP70 binds to lysosomal membranes and acts in the acidic
lysosomal lumen (pH ~ 5)\textsuperscript{11}; many of the cellular effects of HSP70 inhibition by PES and PET-16 relate to the lysosomal function of this protein (Figure 5E)\textsuperscript{4}. The data were fit using a one-site binding model available in the Origin ITC data analysis software (MicroCal ORIGIN V5.0, MicroCal Software, Northampton, MA). The data presented are representative of two or three independent ITC experiments, as indicated. The reported dissociation constants are averages and standard deviations from two or three independent experiments, as indicated. Note, several studies indicate that sigmoidal binding curves are not always obtained with ITC.\textsuperscript{12-17}

Our ITC results contrast with a recently published report that failed to detect a specific interaction between PES and the full-length HSP70 protein using ITC.\textsuperscript{18} We attribute this discrepancy to the disparate experimental conditions employed, including buffer conditions and how the ITC titrations are set up. For instance, Schlecht et al.\textsuperscript{18} performed all titration by injecting PES pre-mixed with ADP (final DMSO concentration of 1\%) into the calorimeter chamber containing the full-length ADP-bound HSP70 protein (final DMSO concentration of 1\%). We found that only when ADP-bound HSP70 was titrated into the calorimeter chamber containing the PES solution (final DMSO concentration of 3\%) was an interaction robust and readily detectable; this may reflect the poor solubility of the PES reagent. In contrast, we found that the interaction was impaired when the PES solution was injected into the sample cell containing the recombinant protein, as was done previously.\textsuperscript{18} We also failed to detect any interaction between HSP70 and PES, when ADP was added to both the calorimeter chamber and titration syringe, as was done in the previous work.\textsuperscript{18} Moreover, the buffer that we used for the ITC analysis does not contain any reducing agent, which can inactivate PES. Thus, by testing several different parameters, and optimizing the ITC protocol conditions, we are readily able to detect a direct, specific interaction between the small molecules and HSP70 as well as between our inhibitors and DnaK. Our finding that PES preferentially targets a "non-ATP-bound" form of HSP70 is consistent with a previous observation that PES inhibits a "non-ATP dependent" form of HSP70 involved in the activation of apo-neuronal nitric oxide synthase.\textsuperscript{19}
Soaking PES and PET-16 into Existing Crystals. We attempted soaking more than three dozen DnaK-SBD-A, DnaK-SBD-B and DnaK-NRLLLTG-A crystals with 1-4 mM PES for time periods between 3 h and 24 h at room temperature before data collection. However, the soaking procedure tended to damage the crystals, and the diffraction spots were smeared. We also incubated more than a dozen DnaK-NRLLLTG-A crystals with varying amount of PET-16 at various intervals at room temperature. However, soaking the DnaK-NRLLLTG-A crystals with PET-16 failed to yield co-crystals of DnaK SBD with PET-16 or DnaK SBD with PET-16 and NRLLLTG.

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Table S2. Data Collection and Refinement Statistics for the Unliganded DnaK Structures

|                  | DnaK-SBD-A | DnaK-SBD-B | DnaK-SBD-C |
|------------------|------------|------------|------------|
| **Data collection** |            |            |            |
| Space group      | P1         | P1         | P2,2,2,1   |
| Cell dimensions  |            |            |            |
| $a, b, c$ (Å)    | 44.66, 69.41, 75.77 | 43.64, 44.83, 63.17 | 92.31, 99.91, 133.72 |
| $\alpha, \beta, \gamma$ (°) | 102.12, 104.20, 92.96 | 77.30, 78.14, 72.96 | 90.00, 90.00, 90.00 |
| Wavelength       | 1.075      | 1.075      | 1.100      |
| Resolution (Å)   | 50-2.36 (2.44-2.36) | 50-1.75 (1.81-1.75) | 45-2.97 (3.08-2.97) |
| $R_{merge}$      | 7.6 (49.7) | 6.3 (49.2) | 10.1 (49.0) |
| $I/\sigma (I)$   | 15.9 (3.0) | 18.6 (2.6) | 19.1 (4.1) |
| Data completeness (%) | 98.0 (97.4) | 96.8 (94.9) | 100.0 (100.0) |
| Redundancy       | 3.9 (3.9)  | 3.8 (3.6)  | 6.6 (6.8)  |
| No. reflections  | 34436      | 43139      | 26253      |
| **Refinement statistics** |            |            |            |
| Resolution (Å)   | 50-2.36 (2.44-2.36) | 50-1.75 (1.81-1.75) | 45-2.97 (3.08-2.97) |
| No. reflections  | 34414      | 43125      | 26126      |
| $R_{work}/R_{free}$ | 21.54/25.82 | 17.62/21.76 | 20.49/24.30 |
| No. atoms        | 6700       | 3930       | 6651       |
| Protein          | 6542       | 3365       | 6563       |
| Ligand/ion       | 15         | 21         | 66         |
| Water            | 143        | 544        | 23         |
| Average B-factor ($\text{Å}^2$) | 61.34   | 33.62      | 83.13      |
| r.m.s. deviations|            |            |            |
| Bond lengths (Å) | 0.0052     | 0.0104     | 0.0041     |
| Bond angles (°)  | 1.065      | 1.319      | 0.965      |

r.m.s., root-mean-square deviation. Values in parentheses are for the highest resolution shell.
### Table S3. Summary of Comparative Analyses

**Cα rmsd (Å) relative to MolA of DnaK-SBD (PDB code 4F01)**

| DnaK-SBD- A | DnaK-SBD- B | DnaK-SBD- C | DnaK-NRLLLTG- A |
|-------------|-------------|-------------|-----------------|
| SBDβ (aa 393-507) [rmsd (Å)] | 0.306 | 0.312 | 0.454 | 0.408 |

**Cα rmsd (Å) relative to MolB of DnaK-SBD (PDB code 4F01)**

| DnaK-SBD- A | DnaK-SBD- B | DnaK-SBD- C | DnaK-NRLLLTG- A |
|-------------|-------------|-------------|-----------------|
| SBDβ (aa 393-507) [rmsd (Å)] | 0.416 | 0.335 | 0.370 | 0.446 |

**Cα rmsd (Å) relative to DnaK-NRLLLTG (PDB code 1DKZ)**

| DnaK-SBD- A | DnaK-SBD- B | DnaK-SBD- C | DnaK-NRLLLTG- A |
|-------------|-------------|-------------|-----------------|
| SBDβ (aa 393-507) [rmsd (Å)] | 0.308 | 0.281 | 0.407 | 0.280 |

**Cα rmsd (Å) relative to DnaK SBDβ (PDB code 1DG4)**

| DnaK-SBD- A | DnaK-SBD- B | DnaK-SBD- C | DnaK-NRLLLTG- A |
|-------------|-------------|-------------|-----------------|
| SBDβ (aa 393-507) [rmsd (Å)] | 2.573 | 2.423 | 2.236 | 2.514 |

**Cα rmsd (Å) relative to DnaK-PET-16 (MolB)**

| DnaK-SBD- A | DnaK-SBD- B | DnaK-SBD- C | DnaK-NRLLLTG- A |
|-------------|-------------|-------------|-----------------|
| SBDβ and SBDα [rmsd (Å)] | 1.242 | 1.209 | 1.176 | 1.742 |
| SBDβ (aa 393-507) [rmsd (Å)] | 0.662 | 0.710 | 0.542 | 0.764 |
Figure S4. PET-16 binding modulates the DnaK structure relative to the unliganded form. (A-C) Left panels, structural comparisons of the DnaK-PET-16 complex with DnaK-SBD (A), DnaK-SBD-A (B) and DnaK-SBDβ (C) structures. The PET-16 compound is shown in red stick format.
Right panels, detailed view of the region near loops LL,1 and Lα,β of the DnaK-PET-16 complex, with residues from DnaK-SBD (A), DnaK-SBD-A (B) and DnaK-SBDβ (C) superimposed. Structural differences noted in loops LL,1 and Lα,β of the DnaK-PET-16 complex and the indicated structures are presented. Note that the same interface that we have shown to be necessary for DnaK-PET-16 complex formation (Figure 4A) is not present in DnaK-SBDβ, as indicated by the shadow representation of the PET-16 molecule.
Table S4. Data Collection and Refinement Statistics for the Substrate-Bound DnaK Structure

| DnaK-NRLLLTTG-A |
|------------------|

### Data collection

- **Space group**: I222
- **Cell dimensions**:
  - \(a, b, c \, (\text{Å})\) 48.24, 57.91, 180.08
  - \(\alpha, \beta, \gamma \, (\text{o})\) 90.00, 90.00, 90.00
- **Wavelength**: 1.100 Å
- **Resolution (Å)**: 45-1.98 (2.05-1.98)
- **\(R_{\text{merge}}\)**: 7.8 (49.8)
- **\(I/\sigma (I)\)**: 24.2 (4.2)
- **Data completeness (%)**: 99.5 (98.7)
- **Redundancy**: 6.5 (6.5)
- **No. reflections**: 18233

### Refinement statistics

- **Resolution (Å)**: 45-1.98 (2.05-1.98)
- **No. reflections**: 18201
- **\(R_{\text{work}}/R_{\text{free}}\)**: 17.37/21.76
- **No. atoms**: 1924
  - **Protein**: 1671
  - **Ligand/ion**: 85
  - **Water**: 169
- **Average B-factor (Å\(^2\))**: 32.33
- **r.m.s deviations**
  - **Bond lengths (Å)**: 0.0152
  - **Bond angles (°)**: 1.599

r.m.s., root-mean-square deviation. Values in parentheses are for the highest resolution shell.
Figure S5. PET-16 binding modulates the DnaK structure relative to the substrate-bound protein. (A and B) Top panels, structural comparisons of the DnaK-PET-16 complex with substrate-bound DnaK-NRLLLTG (A) and DnaK-NRLLLTG-A (B) structures. The PET-16 compound is shown in red stick format. Middle panels, detailed view of the region near loops LL,1 and Lα,β of the DnaK-PET-16 complex, with residues from DnaK-NRLLLTG (A) and DnaK-NRLLLTG-A (B) superimposed. Structural differences noted in loops LL,1 and Lα,β of the DnaK-PET-16 complex and the indicated structures are presented. Bottom panels, detailed
view of Region A and Region B of the DnaK-PET-16 complex, with residues from DnaK-NRLLLTG (A) and DnaK-NRLLLTG-A (B) superimposed.