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Disrupted Hydrogen-Bond Network and Impaired ATPase Activity in an Hsc70 Cysteine Mutant

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Abstract

The ATPase domain of members of the 70 kDa heat shock protein (Hsp70) family shows a high degree of sequence, structural, and functional homology across species. A broadly conserved residue within the Hsp70 ATPase domain that captured our attention is an unpaired cysteine, positioned proximal to the site of nucleotide binding. Prior studies of several Hsp70 family members show this cysteine is not required for Hsp70 ATPase activity, yet select amino acid replacements of the cysteine can dramatically alter ATP hydrolysis. Moreover, post-translational modification of the cysteine has been reported to limit ATP hydrolysis for several Hsp70s. To better understand the underlying mechanism for how perturbation of this noncatalytic residue modulates Hsp70 function, we determined the structure for a cysteine-to-tryptophan mutation in the constitutively expressed, mammalian Hsp70 family member Hsc70. Our work reveals that the steric hindrance produced by a cysteine-to-tryptophan mutation disrupts the hydrogen-bond network within the active site, resulting in a loss of proper catalytic magnesium coordination. We propose that a similarly altered active site is likely observed upon post-translational oxidation. We speculate that the subtle changes we detect in the hydrogen-bonding network may relate to the previously reported observation that cysteine oxidation can influence Hsp70 interdomain communication.

Graphical Abstract

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Accession Codes

The coordinates of wild-type and mutant Hsc70 structures have been deposited in the PDB as entries 6B1I (Hsc70 wild type bound to Mg^{2+} -ADP and P_i), 6B1M (Hsc70 C¹⁷W bound to Mg^{2+} -AppNHp), and 6B1N (Hsc70 C¹⁷W bound to Mg^{2+} -ADP).

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01005. Supplemental Figures 1–5 (PDF)

Members of the 70 kDa heat shock protein (Hsp70) family are molecular chaperones that mediate a variety of protein folding processes within cells. Hsp70s help to facilitate nascent polypeptide folding, the refolding or clearance of destabilized polypeptides, stabilization of mature proteins, and the transport of proteins across organelle membranes.¹ Hsp70s are evolutionarily present from archaea to eukaryotes.^{2,3} In eukaryotes, Hsp70s are localized to major sites of protein folding and assembly, including the cytoplasm, endoplasmic reticulum (ER), and mitochondria.

Hsp70 family members contain two domains, a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD), which are connected via a flexible linker. These two domains are allosterically coupled, wherein the affinity for polypeptide substrates (and chaperone activity) is regulated by the association and/or hydrolysis of the nucleotide (ATP) by the NBD (for recent reviews, see refs 4–6). When the NBD binds ATP, the SBD assumes an open conformation and makes physical contact with the NBD;^{7–9} this open SBD state confers fast on and off rates and a low affinity for the substrate.^{10,11} Upon ATP hydrolysis, the SBD rearranges to close around the peptide substrate,¹² which decreases the off rate and increases the affinity for the substrate. Communication between domains is bidirectional, and binding of a peptide to the SBD also stimulates ATP hydrolysis within the NBD. Several sites of subdomain contact that enable interdomain communication have been identified, including key electrostatic contacts formed between the interdomain linker and the NBD in response to nucleotide binding.^{13–18}

Determining how the nucleotide status within the Hsp70 nucleotide-binding site elicits long-range subdomain rearrangements has been and remains an active and fundamental area of research. Multiple approaches have established that nucleotide-dependent changes within the active site result in a reorientation of the NBD subdomains and long-range structural alterations, which enable interdomain communication.^{15,17,19–23} Structural studies have yielded numerous Hsp70 NBD crystal structures of variable nucleotide status aimed at capturing the changes within the active site upon nucleotide binding; strikingly, these structures show nearly indistinguishable configurations and active site geometries. A comparison of the nucleotide-binding site of 20 structures from the Protein Data Bank (PDB) highlights the high degree of structural similarity among structures, showing an average root-mean-square deviation (RMSD) of 0.32 ± 0.07 Å and a range from 0.21 to 0.48 Å (Figure 1A). Evolutionary tracing of >1600 Hsp70 NBDs shows that residues reported to be critical for nucleotide binding exhibit a robust conservation ranking.²⁴ Corroborating the

structural studies, low-frequency mode analysis of the same residues indicates they are spatially constrained.²⁴ Together, these observations call attention to the conserved and specific set of geometries adopted by Hsp70s that enable proper chaperone activities. These studies also imply that nucleotide binding and hydrolysis likely bring about modest and/or transient changes to the positioning of the conserved and constrained residues within the active site that can effect large rigid-body subdomain rotations. These data suggest that nucleotide-induced changes within the active site are not habitually captured by the X-ray structures.

An intriguing feature of Hsp70 family members is a highly conserved cysteine, juxtaposed to the site of nucleotide binding, that is found in a majority of bacterial, plant, and eukaryotic Hsp70 family members (Figure 1B). The cysteine appears to be dispensable for Hsp70 catalytic function, which is consistent with the cysteine being close to, but not in direct contact with, the bound nucleotide (~10 Å away). An *Escherichia coli* Hsp70 DnaK mutant containing an alanine in place of the conserved cysteine maintains ATPase and peptide-binding activities similar to those of the wild type, and this mutant is able to complement a *dnaK7* deletion strain.^{25,26} Similarly, a cysteine-to-alanine mutant of the yeast ER-localized Hsp70 BiP shows activities comparable to those of wild-type BiP both *in vitro* and *in vivo*.²⁷ Although highly conserved, not all Hsp70s contain this NBD cysteine (Figure 1B), which is again consistent with the dispensability of the cysteine for catalytic activity.

Cysteines are unique in their chemical reactivity, and it has been observed that this cysteine is susceptible to post-translational modification in cells; several bacterial and eukaryotic Hsp70s have been isolated from cell lysates with this conserved NBD cysteine oxidized, including *E. coli* DnaK (Cys15),^{25,28} human Hsc70 (Cys17),²⁹ human BiP (Cys41),³⁰ and yeast BiP (Cys63).^{27,31} For the Hsp70s DnaK and BiP, it has been demonstrated that oxidation of the conserved NBD cysteine limits the rate of ATP hydrolysis.^{27,28,31} Interestingly, further mechanistic studies focused on the ER-localized Hsp70 BiP revealed that cysteine oxidation does not result in an inactive enzyme; in fact, oxidation was shown to enhance the capacity of BiP to limit polypeptide aggregation, even in the absence of ATP hydrolysis.^{27,31,32} Thus, post-translational modification of the NBD cysteine appears to impact interdomain communication. Similarly, additional biochemical studies report that modification of the three NBD cysteines in the cytosolic yeast Hsp70 Ssa1 (including the conserved cysteine) results in an Hsp70 with limited ATPase activity yet an enhanced ability to limit luciferase aggregation.^{33,34} Thus, modification of this conserved cysteine appears to form a means for reversible modulation of Hsp70 activity. We have previously suggested that oxidation of the highly conserved NBD cysteine may influence SBD activity by locally altering the structural arrangement of the active site that, in turn, could influence more global conformational changes in the SBD.²⁷

Curious how oxidation of this conserved cysteine affects the Hsp70 active site, we set out to determine the impact of cysteine modification on the structure of the Hsp70 NBD. Using mammalian cytosolic Hsc70 as a model, we introduced a cysteine-to-tryptophan mutation, which has been shown to recapitulate functional changes associated with oxidation (modification) of the NBD cysteine.²⁷ Here we report a disrupted hydrogen-bond network and loss of magnesium coordination within the active site of the Hsc70 cysteine-to-

tryptophan mutant. We propose that these perturbations account for the loss of ATPase activity associated with oxidation (or tryptophan replacement) of the highly conserved NBD cysteine. We speculate about how the observed changes to the active site may influence Hsp70 interdomain communication. Overall, our data reinforce the importance of the active site architecture of Hsp70 family members for function.

MATERIALS AND METHODS

Protein Expression and Purification

The Hsc70 NBD (residues 5–381) and the near full-length protein (residues 5–554) were constructed by cloning sequences amplified from a mouse cDNA (GenBank accession number BC085486) into pET-28a (EMD Millipore, Billerica, MA). The C¹⁷W point mutations were generated using QuikChange mutagenesis (Agilent Technologies, Santa Clara, CA) and verified by sequencing. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside in *E. coli* BL21 (DE3) at 18 °C. Cells were harvested via centrifugation, resuspended, and sonicated in Ni²⁺-NTA buffer A [25 mM Tris-HCl (pH 8.4), 500 mM KCl, and 20 mM imidazole]. Soluble fractions were collected by centrifugation at 38000g for 1 h; the hexahistidine-tagged proteins were enriched on a Ni²⁺-NTA matrix, and proteins were eluted in Ni²⁺-NTA buffer A supplemented with imidazole to a final concentration of 500 mM. Excess imidazole was removed using a HiPrep 26/10 desalting column (GE Healthcare) that was pre-equilibrated with 25 mM Tris-HCl (pH 7.5), 50 mM KCl, and 10 mM EDTA. Samples were incubated at 4 °C for 12 h to allow for nucleotide release; apoprotein was isolated using a HiTrap Blue HP column (GE Healthcare), and proteins were eluted with a salt gradient (50–1000 mM KCl). Purified proteins were confirmed to be nucleotide-free by high-performance liquid chromatography. Purified protein samples were supplemented with 10% glycerol, concentrated, and subjected to gel filtration on a GE S200 16/60 liquid chromatography column (GE Healthcare) equilibrated with 25 mM HEPES (pH 7.5), 300 mM KCl, and 10% glycerol. Monomer fractions were kept separate and frozen for all biochemical assays, while all protein was pooled and concentrated for crystallography.

ATPase Kinetics

ATP hydrolysis was monitored using an EnzChek phosphate detection kit (Molecular Probes, Eugene, OR) with user-supplied buffer [25 mM HEPES (pH 7.5), 150 mM KCl, 2 mM MgCl₂, and 5% glycerol]. Data were collected from 100 μ L reaction mixtures in half-area 96-well plates (Corning Inc., Corning, NY) using a BioTek Synergy II plate reader set to read at 360 nm. Activity was assayed at 25 °C for a range of Hsc70 protein concentrations (0–7.5 μ M) with 1 mM ATP (Sigma, St. Louis, MO). Raw data were converted to micromolar phosphate using a standard curve, and the initial 10% of substrate conversion was fit to a linear regression to obtain the rate (micromolar P_i per minute). The rate was plotted as a function of enzyme concentration in units of micromolar, and the slope of the linear regression was defined as the turnover number ($k_{\text{cat}} = V/[\text{enzyme}]$).

Differential Radial Capillary Action of Ligand Assay (DRaCALA)

Ligand K_d determination was conducted as previously described.³⁵ In brief, varying concentrations (0.005–10 μ M) of Hsc70 (residues 5–554) were incubated with 5 nM [α -³²P]ATP in 25 mM HEPES (pH 7.5), 100 mM KCl, 2 mM MgCl₂, and 0.5 mM DTT for 10 min. Samples (4 μ L) were spotted onto dry nitrocellulose. The ligand diffusion signal was imaged with a phosphorimager (GE Healthcare), and signal intensities were quantitated using ImageJ.³⁶ Data were normalized between 0 and 1 and fit to a saturation-binding model [$Y = Y_{\max} \times X/(K_d + X)$] to determine the ATP dissociation constant.

Hsc70 Crystallization, Structure Determination, and Modification Modeling

Hsc70 wild-type and C¹⁷W crystals were obtained from the NBD fragment (residues 5–381) through sitting drop vapor diffusion. Protein (10–30 mg/mL) was incubated with 5 mM nucleotide and 5 mM MgCl₂ for 20 min at 25 °C, mixed with an equal volume of reservoir solution, and incubated at 20 °C, where crystals developed within 12 h in a reservoir solution of 0.2 M sodium chloride, 0.1 M Tris (pH 8.5), and 25% (w/v) polyethylene glycol 3350 (Hampton Research, Aliso Viejo, CA). Crystals were subsequently optimized via hanging drop vapor diffusion, yielding crystals suitable for diffraction experiments in 0.2 M sodium chloride, 0.1 M Tris (pH 9), and 24% (w/v) polyethylene glycol 3350 for wild-type Hsc70 and 0.2 M sodium chloride, 0.1 M Tris (pH 7.4), and 26% (w/v) polyethylene glycol 3350 for Hsc70 C¹⁷W. Upon being harvested, crystals were soaked under the previously mentioned conditions supplemented with 25% glycerol as a cryoprotectant for 5 min and frozen in liquid nitrogen.

X-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS). Data processing and scaling were completed with X-ray Detector Software (XDS) and the CCP4 software suite.^{37,38} Phases were attained through molecular replacement methods using the PHENIX software package and the coordinates of Hsc70 [Protein Data Bank (PDB) entry 4H5R] as a search model.^{39,40} Refinements were conducted in PHENIX and COOT for producing the final model.^{39,41} Data collection and model statistics are summarized in Table 1. Figure illustrations, RMSD calculations, and distance measurements were made in Pymol (version 1.8.4, Schröffding, LLC). The previously mentioned software packages were accessed through SGrid (www.sgrid.org).⁴²

Modeling of sulfonic acid (–SO₃) and the glutathione adduct was achieved by replacing C¹⁷ in the wild-type structure from this study with the corresponding modification using COOT, creating a structure factor model with phenix.fmodel, and conducting simulated annealing and geometry minimizations using PHENIX to attain the final models.

Thermal Shift Assay

A fluorescence-based thermal shift assay using SYPRO Orange (Invitrogen, Carlsbad, CA) was used to generate protein melting curves and calculate T_m values.⁴³ Each reaction mixture contained 2 μ M Hsc70, 2 mM nucleotide, 4 mM MgCl₂, and 5 $\times$ SYPRO Orange in 25 mM HEPES (pH 7.5), 300 mM KCl, and 10% glycerol. A 20 μ L reaction mixture was read in a MicroAmp 384-well plate (Applied Biosystems, Foster City, CA) using a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). The protocol was initiated

with a 5 min incubation at 10 °C followed by an increase in temperature at a rate of 0.03 °C/s to 95 °C. Excitation and emission wavelengths were 470 ± 15 and 586 ± 10 nm, respectively. T_m values were defined by calculating the temperature at which the second derivative of the thermal unfolding curve was equal to zero using GraphPad Prism (GraphPad, La Jolla, CA).

Rhodanese Aggregation Assay

Denatured rhodanese (Sigma) was prepared and stored as described previously.²⁷ Rhodanese aggregation was initiated by diluting thawed denatured rhodanese to 1 μ M in 0.1 mL of buffer with a final composition of 25 mM HEPES (pH 7.4), 120 mM KCl, 5 mM MgCl₂, 1 mM ATP, 5% glycerol, and 8 μ M Hsc70 (residues 5–554) or 8 μ M BSA. Rhodanese aggregation was monitored by following the scattering of light at 320 nm over time. The maximal light scattering at 10 min observed in the absence of the Hsc70 chaperone (in the presence of BSA) was set to a value of 1.0, and all data were normalized to these maximal light scattering values. Assays reflect six replicates performed over three independent experiments.

Quantification and Statistical Analysis

Data fitting, including baseline corrections, normalization, calculation of the mean and standard deviation, and statistical tests, were conducted using GraphPad Prism (version 6.0h). Pairwise distance difference matrices for crystal structures within the same space group were calculated using the SuperPose web server (<http://wishart.biology.ualberta.ca/SuperPose/>).⁴⁴ Individual difference matrices were visualized using matplotlib.contour.⁴⁵ Figure legends indicate the number of biological and technical replicates and the entity plotted. Data baseline correction and normalization, where applied, were indicated in the corresponding method section and in the graph axis labels. No explicit power analysis was used.

RESULTS

The Hsc70 C¹⁷W Mutation Impairs ATP Hydrolysis but Not ATP Binding

We first assessed how a cysteine-to-tryptophan mutation impacts ATP hydrolysis in mammalian Hsc70. We measured phosphate release kinetics for both recombinant wild-type and mutant Hsc70 C¹⁷W proteins (residues 5–554). The k_{cat} value for wild-type Hsc70 was determined to be 0.096 ± 0.002 min⁻¹ (Figure 1C), which is consistent with the slow turnover of the nucleotide by Hsp70s and the wide range of published k_{cat} values reported for unstimulated Hsc70 activity (0.072 – 0.86 min⁻¹).^{46–48} Hsc70 C¹⁷W exhibited severely impaired catalytic activity with a measured k_{cat} of 0.004 ± 0.001 min⁻¹ (Figure 1C). The loss of ATPase activity observed for the Hsc70 C¹⁷W mutant is consistent with previous studies demonstrating that replacement of the conserved Hsc70 cysteine with lysine or arginine results in proteins with extremely low or no measurable hydrolytic activity, respectively.⁴⁹ These data are consistent also with substitutions of the positionally analogous cysteine in the ER-localized Hsp70 BiP, wherein a cysteine-to-tryptophan replacement mutant showed negligible ATPase activity.^{27,50} The limited ATP turnover by these mutants is likely a consequence of the introduction of a large side chain at the cysteine position; prior

studies with BiP show a cysteine-to-alanine mutant maintains wild-type ATPase activity.^{27,31,50} Measurements of ATP turnover by the ATPase domain alone (Hsc70 residues 5–381) suggest that it is a disruption in NBD activity that accounts for the limited ATP hydrolysis by the C¹⁷W mutant. The k_{cat} value for wild-type Hsc70 NBD was determined to be $0.088 \pm 0.001 \text{ min}^{-1}$ versus a k_{cat} of $0.0021 \pm 0.0004 \text{ min}^{-1}$ for Hsc70 C¹⁷W (Supplemental Figure 1).

We hypothesized that the large side chain of tryptophan effected ATP hydrolysis by altering nucleotide affinity. To test the relative binding affinities of wild-type and mutant Hsc70s, we conducted a differential radial capillary action of ligand assay (DRaCALA).³⁵ Various amounts of near full-length Hsc70 protein (confirmed to be devoid of bound nucleotide) were mixed with a constant amount of ligand ($[\alpha\text{-}^{32}\text{P}]\text{ATP}$), and the protein/ATP mixture was spotted onto dry nitrocellulose to separate the free ligand from protein–ligand complexes; ligand associated with protein was immobilized when spotted onto nitrocellulose, while free ligand could diffuse radially through capillary action. Via quantification of the amounts of free and bound ligand across a range of protein concentrations, a binding constant was determined.³⁵ We found that wild-type and C¹⁷W proteins showed comparable affinities for ATP; dissociation constants (K_d) of 0.21 ± 0.04 and $0.16 \pm 0.06 \mu\text{M}$ were measured for the wild type and the C¹⁷W mutant, respectively (Figure 1D). The K_d values obtained from our DRaCALA experiments fall within the wide range of values for dissociation constants reported for wild-type Hsc70 in the literature ($0.042\text{--}9.5 \mu\text{M}$).^{10,48,51–54} Consistent with our observations with Hsc70 C¹⁷W, an Hsc70 C¹⁷K mutant also retains the capacity to bind nucleotide; indeed, the measured K_m for ATP hydrolysis and the K_d (assessed by filter binding) for Hsc70 C¹⁷K indicate an interaction with the nucleotide that is tighter than that of the wild type.⁴⁹

Crystal Structures of Hsc70 Wild Type and C¹⁷W Reveal a Mechanism for Loss of ATPase Activity

To further explore how a C¹⁷W mutation might impact Hsc70 function as an ATPase, we determined three crystal structures of the human/mouse Hsc70 NBD (residues 5–381; Hsc70 residues 5–381 are identical for both mouse and human Hsc70). Wild-type protein crystals were isolated bound to Mg^{2+} ADP and P_i and diffracted X-rays to $2.3 \text{ \AA}$ (Figure 2A). Crystals were obtained for the mutant Hsc70 C¹⁷W protein bound to both Mg^{2+} ADP (Figure 2B) and Mg^{2+} AppNHp (Figure 2C), and crystals diffracted X-rays to 1.8 and $1.9 \text{ \AA}$, respectively. All three structures were in space group $P2_1$ with two molecules in the asymmetric unit (Table 1). An α -carbon backbone overlay revealed an overall global similarity between the wild-type and mutant proteins; the C¹⁷W Mg^{2+} ·ADP and C¹⁷W Mg^{2+} ·AppNHp structures both have RMSD values of 0.41 when compared to that of the wild type (Figure 2D). However, a search for more subtle differences between the three structures uncovered local differences in the magnesium· nucleotide coordination by the wild-type and mutant proteins (Figure 3).

Focusing initially on our wild-type Mg^{2+} ·ADP and P_i structure, we observed a strong correlation of active site geometries with previously published Hsp70 structures.^{40,55–59} A magnesium ion forms a bidentate complex with the β -phosphate of ADP and the hydrolyzed

γ -phosphate. The bidentate complex is stabilized by water molecules, which are coordinated by D¹⁰, K⁷¹, E¹⁷⁵, and D¹⁹⁹ (Figure 3A).⁶⁰ The location of K⁷¹ within the active site is also maintained across the various structures (Figure 3A); K⁷¹ is proposed to facilitate the nucleophilic attack on ATP via positioning a water molecule in line with the γ -phosphate.⁶¹ The prehydrolysis state of GRP78, visualized with GRP78 bound to the nonhydrolyzable analogue Mg²⁺ AppCHp (PDB entry 5F2R) (Figure 3B),⁵⁹ shows also the same active site configuration we observe for the Hsc70 Mg²⁺ ADP and P_i structure (Figure 3A), reinforcing the high degree of active site conservation between Hsp70 members.

Switching our attention to the C¹⁷W mutant structures, we observed distinct changes within the active site when compared against that of the wild-type protein. Specifically, the location of the magnesium ion was altered in the C¹⁷W structures (Figure 3C, D). In the wild-type structure, a water molecule (denoted H₂O[‡]) hydrogen bonds with the backbone carbonyl of D³⁶⁶, the carboxylic acid of D¹⁰, the α -phosphate of the nucleotide, and an additional water molecule (Figure 3A, B). We found the tryptophan mutation introduces a steric hindrance in the vicinity of the H₂O[‡] molecule, which results in the absence of the H₂O[‡] in the structures of both C¹⁷W Mg²⁺·ADP and C¹⁷W Mg²⁺·AppNHp (Figure 3C, D). Without the H₂O[‡], the canonical hydrogen-bonding network in the active site is disrupted. Magnesium coordination is maintained in the C¹⁷W Mg²⁺·ADP structure (in the absence of the H₂O[‡]) through a direct interaction of the magnesium with the α -phosphate of ADP, which causes the magnesium ion to recede from the catalytic residues (Figure 3C). Alternatively, in the C¹⁷W Mg²⁺·AppNHp structure, the carboxylic acid moiety of D¹⁰ engages the magnesium ion (Figure 3D); this arrangement is in contrast to other Hsp70 structures, where the interaction of D¹⁰ with magnesium is stabilized through a water molecule [or potassium (see Discussion)]. Calculated $F_o - F_c$ omit maps clearly resolve electron density for the C¹⁷W mutation at 4 σ in both mutant structures (Supplemental Figure 2). Additionally observed for the C¹⁷W structures is the loss of a critical water-bridge hydrogen bond between the Mg²⁺·nucleotide and residues E¹⁷⁵, K⁷¹, and D¹⁹⁹ (water is denoted H₂O*) (Figure 3A–D). This water-bridged hydrogen bond has previously been implicated in a path for interdomain allostery, wherein the nucleotide-bound state of the NBD is relayed to the SBD via a proposed proline switch.⁶²

Given that our wild-type and C¹⁷W structures were of different nucleotide-bound states, we were curious whether the changes in Mg²⁺ related to the identity of the coordinated nucleotide or were a consequence of the introduction of a tryptophan at position 17. To determine if the displacement of the magnesium ion in our mutant structures was a unique active site configuration, we culled wild-type and mutant Hsp70 structures with an associated magnesium·ligand and compared these structures with those of our C¹⁷W mutants. Structures from bacteria and eukaryotes with various ligands (ADP, ATP, ADP and P_i, AppCHp, ADP·VO₄^{3−}, P_i, or 7-deazaATP) and a magnesium ion were selected for analysis, and these structures were aligned with our wild-type Hsc70 Mg²⁺·ADP and P_i structure (PDB entry 6B1I). The 25 aligned crystal structures (including PDB entries 6B1I, 6B1M, and 6B1N from this study) exhibited a high degree of similarity, with RMSD values for eukaryotic Hsp70s ranging from 0.34 to 0.70 Å and an RMSD value for bacterial DnaK of 3.3 Å (Figure 4A). Inspection of the Mg²⁺·nucleotides from the aligned structures indicated a strong preponderance of magnesium ions at the same location independent of the

type of bound nucleotide; only the magnesium ions from our C¹⁷W structures deviated from the group (Figure 4B). Magnesium displacement was calculated by measuring the distance between the magnesium ions using our wild-type structure as the reference. A modest mean displacement of 0.27 ± 0.03 Å was calculated for the magnesium ions in the 22 structures with similarly positioned magnesiums (Figure 4C). In contrast, the magnesium ions in the C¹⁷W mutants were significantly displaced by distances of 2.1 and 1.9 Å in the Mg²⁺·AppNHp and Mg²⁺·ADP structures, respectively (Figure 4C). The altered positioning of the magnesium ions in our two Hsc70 C¹⁷W structures is particularly striking given the structural conservation of the active site (and magnesium positioning) across a range of Hsp70 structures (Figure 1A), including two Hsc70 structures from active site mutants (PDB entries 1BA1 and 1KAX) that showed no significant perturbation of the magnesium positioning relative to that of wild-type Hsc70^{49,61} (Figures 1A and 4C).

Thermal Stability of Wild-Type and C¹⁷W Proteins Are Affected by the Nucleotide and Magnesium

In light of the distinct catalytic magnesium localization in the wild-type and C¹⁷W mutant crystal structures, we sought to address if nucleotide and/or magnesium coordination differences impacted the relative thermal stabilities of the wild-type and mutant proteins in the presence of ligand. Using an established fluorescence-based thermal shift assay,⁴³ we monitored first the influence of the nucleotide on the thermal stability of near full-length wild-type Hsc70 (residues 5–554). We observed that nucleotide addition (with or without magnesium) increased the measured T_m . Addition of ADP or ATP in the absence of magnesium increased the mean T_m value by 7 °C, relative to that of the apoenzyme (Figure 5A, left). Addition of nucleotide and magnesium further stabilized the Hsc70 protein, resulting in a >10 °C increase in T_m relative to that of apo Hsc70 (Figure 5A). Interestingly, a nucleotide-specific stabilization effect was observed for the wild-type proteins in the presence of nucleotide and magnesium, with a T_m of 3 °C between Mg²⁺·ADP and Mg²⁺·ATP (Figure 5A, center and right).

Turning our attention to the C¹⁷W protein, we observed first an overall decrease in thermostability relative to that of the wild-type protein; the mean T_m for apo C¹⁷W was 35.5 °C relative to 47 °C observed for the apo wild type (Figure 5). Addition of nucleotide (with or without magnesium) significantly increased the C¹⁷W protein stability (Figure 5B). It is worth noting that the mutant protein in its most stable state (in the presence of magnesium and nucleotide) was still relatively thermo-labile, exhibiting stability similar to that of the wild-type protein in its least stable (apo) state (Figure 5). The addition of nucleotide alone to the C¹⁷W protein increased the thermostability by ~7 °C, relative to that of the apoprotein (Figure 5B). Both ADP and ATP with magnesium stabilized the C¹⁷W protein by a further ~6 °C, relative to that of nucleotide alone (Figure 5B, center). As observed with wild-type Hsc70, an increase in stability was conferred by Mg²⁺·ATP relative to Mg²⁺·ADP. Interestingly, in contrast to what was observed in the presence of magnesium (Figure 5B, center), in the absence of magnesium, the ADP-bound protein showed an increase in stability relative to that of the ATP-bound form (Figure 5B, left). Together, these data demonstrate a significant thermal shift conferred by the nucleotide for both wild-type and Hsc70 proteins, which is enhanced by the presence of magnesium. The increased

stability of nucleotide-bound C¹⁷W in the presence of magnesium (relative to nucleotide alone) is consistent with the coordination of magnesium in the C¹⁷W active site structures, although the aberrant coordination appears to be disruptive for catalytic function (Figure 1). These data suggest also that the presence of a tryptophan at position 17 somehow alters the positioning of the nucleotide in the absence of magnesium, which results in a nucleotide-specific difference in stability that is not observed for the wild-type protein.

An overall decrease in thermostability seen for the near full-length C¹⁷W mutant (relative to that of the wild-type protein) was observed also for the Hsc70 C¹⁷W NBD protein; the mean T_m for apo C¹⁷W NBD was 34.8 °C relative to the value of 46.5 °C observed for apo wild-type NBD (Supplemental Figure 3 and Figure 5). These data suggest that the decrease in thermostability of the NBD accounts primarily for the lower thermostability observed for the near full-length C¹⁷W mutant. As seen for the near full-length proteins, nucleotide addition (with or without magnesium) increased the measured T_m for the wild-type and C¹⁷W NBD proteins. The increase in T_m was slightly less pronounced for the NBD proteins than for their near full-length counterparts; for example, addition of ADP or ATP in the absence of magnesium increased the mean T_m value of the wild-type Hsc70 NBD by ~5 °C (vs 7 °C for the near full-length protein) relative to those of the corresponding apoenzymes (Figure 5 and Supplemental Figure 3). The nucleotide-specific stabilization effect seen in the presence of magnesium for the near full-length Hsc70s (Figure 5) was observed also for wild-type NBD proteins, but again, the degree of stabilization was more modest: for example, a T_m of 1.5 °C between the wild-type Mg²⁺·ADP and Mg²⁺·ATP NBD forms versus a T_m of 3 °C for the full-length Hsc70 states (Supplemental Figure 3). Together, these data suggest that the stability of the NBD contributes strongly to the overall Hsc70 thermostability. These data also suggest that the presence of the SBD confers some additional (albeit modest) stability to both wild-type and C¹⁷W mutant proteins in the presence of the nucleotide.

Long-Range Distance Fluctuations between Wild-Type and C¹⁷W Structures

To quantitatively assess whether the C¹⁷W mutation introduced long-range structural changes, we compared wild-type and mutant structures using a pairwise distance difference matrix. For these comparisons, each structure is represented as a matrix composed of vectors that together represent the distance between all α -carbon atoms in a given structure. The matrices for wild-type and mutant structures can be subtracted to elucidate distance differences between structures.⁴⁴

Comparing the wild-type structure with either C¹⁷W·AppNHp or C¹⁷W·ADP structures, we observed that subdomain IIB is closer to both the IA (Figure 6, orange arrows) and IB (Figure 6, pink arrows) subdomains in the C¹⁷W mutants. In addition, the distance of a region near residue 32 of subdomain IA from all the NBD subdomains in the C¹⁷W structures was found to be altered relative to what is observed for the wild type (Figure 6, red arrow). Similar directional distance changes were seen for the C¹⁷W·AppNHp and C¹⁷W·ADP structures, yet subdomain IIB was closer to both subdomains IA and IB in the AppNHp-bound structure (Figure 6A). Additional distance changes unique to the C¹⁷W·AppNHp structure were observed in the proximity of residues 191 and 214 (located in subdomain IIA) (Figure 6A, red arrows). Interestingly, these residues are close (8–15 Å) to

the hydrophobic surface cleft in the NBD that serves as a docking site for the interdomain linker.^{7–9,18} In the ATP-bound state, linker docking stabilizes the open SBD state, and the presence of the docked linker has been implicated in allosteric communication between the NBD and SBD.⁶³ However, unlike the changes in subdomain IIB positioning that were observed for both Hsc70 C¹⁷W structures (independent of nucleotide state), here we cannot confirm that these changes relate specifically to the C¹⁷W mutation; we cannot rule out the possibility that the relative distance changes in the vicinity of residues 191 and 214 are a consequence of the type of bound nucleotide (AppNHp vs ADP). Prior NMR and modeling studies of the NBD of *Thermus thermophilus* DnaK revealed a substantial change in rotation of domains IIA and IIB (relative to IA) when switching between the AMPPNP and ADP states, which resulted in a change in the accessibility of the hydrophobic surface cleft.¹⁵

Performing a similar analysis between the structures of a previously published Hsc70 C¹⁷K mutant bound to Mg²⁺·ADP (PDB entry 1BA1) and wild-type Hsc70 bound to Mg²⁺·ADP and P_i (PDB entry 3HSC) from the same space group^{49,55} revealed no obvious distance changes (Supplemental Figure 4A), highlighting the significance of the altered positioning for the IIB subdomain (in relation to IA and IB) observed for the C¹⁷W structures. The similar distance matrices for these structures also highlight the negligible influence of the nucleotide-bound state (ADP vs ADP with P_i) in this analysis (relevant also for the comparisons in Figure 6B). Structural alignments of the C¹⁷K and C¹⁷W structures did reveal a subtle 2 Å shift in residues 55–62 located in subdomain IB and residues 213–216 in subdomain IIA (Supplemental Figure 4B).

DISCUSSION

Functional Outcomes for an Altered Active Site in an Hsc70 C¹⁷W Mutant

On the basis of a comparative analysis of wild-type and mutant crystal structures, we conclude that the steric hindrance imposed by introduction of a tryptophan at position 17 results in displacement of a water molecule (denoted H₂O[‡]), disrupting the hydrogen-bond network within the Hsc70 active site (Figure 4A). We suggest that a consequence of the altered hydrogen-bond network is the 2 Å displacement of the catalytic magnesium in Hsc70 C¹⁷W from its “standard” position (Figures 3 and 4), the standard position being defined as the magnesium coordination observed in the 22 representative Hsp70 structures collected from the PDB (Figure 4C). We propose that the observed magnesium displacement accounts for the decrease in the rate of ATP hydrolysis observed for Hsc70 C¹⁷W (Figure 1C).

Given the proximity of the conserved cysteine to the bound nucleotide, we originally anticipated that the cysteine-to-tryptophan mutation might hinder access of the nucleotide to the ligand-binding pocket and/or alter nucleotide affinity. However, we found the C¹⁷W mutation had no detectable impact on the Hsc70 ATP *K_d*. In addition, we observed occupancy of the active site with a nucleotide (Mg²⁺·ADP or Mg²⁺·AppNHp) in two independent Hsc70 C¹⁷W structures (Figure 3C, D). Our observations are consistent with prior studies of an Hsc70 C¹⁷K mutant, which exhibited tighter binding to the nucleotide (relative to that of the wild type) and was crystallized in complex with ATP, ADP, and P_i.⁴⁹ These authors similarly concluded that the reduced hydrolytic rates observed for Hsc70 C¹⁷K reflected a deficiency in the ATPase cycle after nucleotide binding.⁴⁹ It is interesting

to note that while both Hsc70 C¹⁷K and C¹⁷W mutants show a loss of ATPase activity, our structural data suggest a more severely compromised active site in the C¹⁷W mutant (relative to the published C¹⁷K structure).⁴⁹ Notably, studies of different amino acid substitutions of the conserved BiP cysteine in yeast revealed also the strongest phenotypes with a BiP C⁶³W allele, relative to a BiP C⁶³K mutant.⁵⁰

Two potassium-binding sites have been characterized in the Hsc70 active site, identified by anomalous difference Fourier peaks.⁵⁶ We observed electron densities at the positions previously assigned to potassium ions, yet we were unable to unambiguously determine whether potassium ions were present in our structures. We instead used water molecules to model the corresponding densities; the water molecules modeled at the previously determined “site 1” potassium site are denoted with pink halos (Figure 3). It is interesting to note that the displaced magnesium observed in our C¹⁷W structures is hydrogen-bonded to the water modeled at one of the characterized potassium-binding sites; this interaction likely aids the stable association of Hsc70 C¹⁷W with the nucleotide. In the Mg²⁺·ADP structure, the aforementioned water molecule remains in the same location as in the wild-type structure (Figure 3C); in the Mg²⁺·AppNHp structure, the water molecule shifts 1.1 Å toward the carboxylic acid of D¹⁰ (Figure 3D). Given the importance of potassium for optimal Hsc70 ATPase activity,⁶⁴ we propose that the changes in the potential placement and/or coordination of potassium may also contribute to the observed decrease in the rate of ATP turnover for the Hsc70 C¹⁷W mutant.

As mentioned in the introductory section, the conserved NBD cysteine that is the focus of this study is susceptible to post-translational modification in cells. Several Hsp70 family members have been isolated from cells with the conserved cysteine oxidized. In the case of Hsc70, a quantitative proteomic study identified Hsc70 C¹⁷-containing peptides modified with either sulfinic (-SO₂H) or sulfonic (-SO₃) acid in cell lysates treated with hydrogen peroxide.²⁹ It has previously been demonstrated for the Hsp70 BiP that a cysteine-to-tryptophan mutation recapitulates the *in vivo* phenotypes and *in vitro* activity changes associated with oxidation of the conserved BiP cysteine by peroxide or glutathione;²⁷ we anticipate that oxidation of the Hsc70 cysteine could elicit similar changes to the active site geometry as we report here for the C¹⁷W mutant.

To lend support to our speculation, we modeled two characterized Hsp70 cysteine adducts, sulfonic acid (-SO₃) and a glutathione, into the wild-type structure from this study (Figure 7) (see Materials and Methods for modeling details). A display of the solvent-excluded surfaces of cysteine and tryptophan highlights the increase in side-chain size observed in the mutant structure relative to that of the wild type (Figure 7B vs Figure 7A). A modeled sulfonic acid moiety exhibits a size and a position similar to those observed in the Hsc70 C¹⁷W mutant (Figure 7C). In support of a similar impact for a sulfonic acid addition and a tryptophan substitution, the modeling suggests that a sulfonic acid would partially occupy the location of the H₂O[‡] found in the wild-type structure (Figure 7C); we propose modification of the cysteine with peroxide would likely displace the H₂O[‡] as we observed for Hsc70 C¹⁷W (Figure 7B). Modeling of a glutathione adduct suggests the potential for an active site even more perturbed than that with sulfonic acid or tryptophan; the tripeptide glutathione is a much larger molecule and results in a higher occupancy of the active site

(Figure 7D). Our glutathione adduct model suggests that the active site of glutathionylated Hsc70 can still accommodate the nucleotide (Figure 7D); however, we cannot discount that the large size of this modification and the variable conformations it may adopt could impede nucleotide binding. Studies with recombinant glutathionylated DnaK have demonstrated a weaker (but detectable) binding of glutathione-modified DnaK to ATP-agarose,²⁸ relative to the binding observed with unmodified or deglutathionylated DnaK.

Thermal shift data for the near full-length protein are consistent with, and further validate, our structural data; the thermal shift data indicate both a destabilized Hsc70 C¹⁷W protein (relative to the wild-type protein) and a capacity of the mutant protein to bind ATP. We observed the global thermostability of C¹⁷W was compromised when it was compared to the wild-type protein (Figure 5 and Supplemental Figure 3), and we suggest that the loss of thermostability reflects (at least in part) the disrupted active site visualized in the crystal structures. Consistent with our observations, and with the potential similarity between a tryptophan mutation and cysteine oxidation, it has been reported that glutathionylated DnaK shows a complete loss of the first thermal transition normally seen with unmodified DnaK;²⁸ this first transition has been attributed to the unfolding of the DnaK NBD. It is worth noting that several studies using differential scanning calorimetry report a multistate denaturation curve for the Hsp70s (e.g., like the aforementioned DnaK study²⁸), yet the single transition we observed for Hsc70 is consistent with studies that used a thermoffuor method similar to what we describe herein.^{65,66} Our data also indicate that nucleotide (ADP or ATP) association increases the stability of both wild-type and C¹⁷W proteins (Figure 5). Similar to our observations, the addition of Mg·ATP to glutathionylated DnaK restored thermal transition values equivalent to those observed with unmodified DnaK,²⁸ yet unlike what was reported for DnaK, we found that while Mg·ATP conferred a significant level of thermostability to the Hsc70 C¹⁷W mutant, the level of stability was not comparable to that seen with wild-type Hsc70 (Figure 5). We also observed a subtle difference in the stability of the near full-length Hsc70 C¹⁷W mutant conferred by ADP and ATP in the absence of magnesium, the significance of which is unclear (Figure 5B); ATP and ADP bestowed stability similar to that of wild-type Hsc70 under the same magnesium-independent conditions (Figure 5A). These data likely reflect distinctions in nucleotide positioning within the wild-type and mutant active site, in the absence of magnesium, that remain to be elucidated. It is interesting that this distinction was not observed for the C¹⁷W NBD protein (Supplemental Figure 3), and the difference seen for the full-length C¹⁷W protein could reflect a change in the stability of the SBD in the presence of Mg²⁺·ADP and Mg²⁺·ATP.

Potential Implications for Cysteine Modification on Interdomain Communication

It has been established for the ER-localized Hsp70 BiP that loss of ATPase activity is only one consequence of cysteine mutation or cysteine oxidation. These prior studies demonstrate that cysteine oxidation (or alternatively a cysteine-to-tryptophan mutation) confers not only a loss of ATPase activity but also an enhanced association with polypeptides.^{27,31} A similar functional outcome has been reported also for the small molecule MKT-077 (and its derivatives), which simultaneously inhibits ATPase activity and enhances the binding of Hsp70 to unfolded protein substrates.^{67,68} Reminiscent of the location of the redox-sensitive cysteine, a hydrophobic binding pocket for MKT-077 and its analogues has been mapped,

which is adjacent to (but not overlapping with) the nucleotide-binding cleft.^{67,69} It has been suggested that MKT-077 and its analogues preferentially bind to Hsc70 in its ADP-bound state;⁶⁹ binding is proposed to stabilize the ADP-bound form, allowing for a closed SBD conformation and increased substrate affinity. Models do not place Hsc70 C¹⁷ in contact with MKT-077, yet residues in the proximity of C¹⁷ are proposed to stabilize MKT-077 within its proposed hydrophobic binding pocket.⁶⁹ It is tempting to speculate that structural perturbations we observe in the active site of the Hsc70 C¹⁷W mutant may effect a stabilization of the ADP-bound form and affinity for polypeptides, like observed for MKT-077 and suggested by prior studies with oxidized BiP. Consistent with such a model, we observed that the Hsc70 C¹⁷W mutant showed an increased capacity to limit aggregation of a model polypeptide (rhodanese) relative to wild-type Hsc70 (Supplemental Figure 5). This observation suggests that a consequence of the C¹⁷W mutation is a higher affinity of the Hsc70 for polypeptides and/or a weakened ability of the Hsc70 to release bound polypeptides, either of which could enhance the ability of the Hsc70 C¹⁷W mutant to limit the self-association and aggregation of rhodanese.

Interestingly, structural changes in the C¹⁷W mutant are observed at residues previously implicated in interdomain communication, suggesting a potential means for cysteine modification to alter SBD peptide-binding activity. The C¹⁷W proteins show a loss of a critical water-bridge hydrogen bond, relative to wild-type Hsc70 (Figure 3, H₂O*). It has been proposed previously that the interactions among H₂O*, Mg²⁺, nucleotide, and residues E¹⁷⁵ and K⁷¹ change depending on nucleotide status; these nucleotide-dependent changes are suggested to induce a positional shift in the peptide backbone, which is relayed through select NBD residues to ultimately elicit a change in SBD conformation.⁶² Moreover, the conformation of these residues is proposed to change in response to signals relayed from the SBD to the NBD upon peptide association.⁶² Interestingly, mutation of the equivalent glutamic acid in DnaK results in a loss of ATP hydrolysis activity and slower peptide dissociation,⁶² similar to what has been proposed for BiP oxidation. Defects in interdomain coupling for an Hsc70 E¹⁷⁵S mutation have also been observed, and these defects could be rescued by a secondary mutation in the peptide-binding domain (an E⁵⁴³K mutation).⁷⁰ These latter observations were interpreted as a rescue of an Hsc70 E¹⁷⁵S mutant protein “locked” in an ADP-like state (i.e., unable to undergo the transition to the ATP-induced form) by a secondary mutation that could restabilize the ATP-induced state.⁷¹

Long-range distance changes also support our speculation that the C¹⁷W mutation alters the NBD domain conformation, which may, in turn, affect interdomain allostery. The pairwise distance matrices comparing the wild-type and C¹⁷W structures indicate a decreased distance between subdomain IIB and subdomains IA and IB in the C¹⁷W mutant relative to those in wild-type Hsc70 (Figure 6, orange and pink arrows). Rotation of subdomain IIB has been correlated with nucleotide release and opening of the nucleotide-binding cleft, which precedes polypeptide release.¹⁷ The closer positioning of subdomain IIB in the C¹⁷W structures could reflect changes in NBD dynamics that may, in turn, impact SBD dynamics. In addition, the matrices revealed structural perturbations in the C¹⁷W·AppNHp structure near known allosteric sites, including two residues, 191 and 214, found on loops that are poised within 8–15 Å of the interdomain linker-docking site.⁹ Whether these changes are a consequence of the C¹⁷W mutation or if they are related to the AppNHp nucleotide remains

to be further explored. The distance changes observed in the C¹⁷W mutant structures are unique to the tryptophan substitution and were not observed for the C¹⁷K mutant. Conducting an analogous comparison between C¹⁷K·ADP (PDB entry 1BA1) and the wild type bound to ADP and P_i (PDB entry 3HSC) reveals no differences between different nucleotide-bound states or the introduction of a lysine substitution (Supplemental Figure 4).^{49,55} As mentioned above, these observations are consistent with our prior data relating to BiP, which demonstrated a more severe effect on chaperone activities for a BiP C⁶³W mutation than for a C⁶³K mutation.⁵⁰

Taken together, these data lead us to propose that the altered hydrogen bonding we observed in the Hsc70 C¹⁷W mutant (where the connectivity between Mg²⁺-nucleotide and E¹⁷⁵ is also disrupted) could bring about changes to Hsc70 domain communication, affecting peptide association while also limiting ATP hydrolysis. Such a mechanism would be consistent with the prior observations with the yeast Hsc70 ortholog Ssa1, which showed both a decrease in the ATPase rate and an enhanced ability to limit polypeptide aggregation upon cysteine alkylation.^{33,34} Future studies focused on the dynamics of full-length Hsc70 should shed light on if and/or how cysteine alteration impacts interdomain communication. Given the accumulating accounts of Hsp70 family members that acquire modifications at the active site cysteine, we propose that the molecular details presented here may suggest a general mechanism for modulation of Hsp70 activity through post-translational modification that can be extended to other Hsp70s and their cellular activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hsc70 cysteine mutation inhibits ATP hydrolysis but not ATP binding. (A) Hsp70 family structural overlay of residues within 4 Å of adenosine nucleotides. RMSD calculations were completed for the indicated Hsp70 members against the wild-type Hsc70 structure from this study.^{40,49,55–59,61,72–74} Structures with amino acid replacements at T¹³, C¹⁷, or K⁷¹ were included in the alignment, and mutations are indicated. The numbering corresponds to the mouse Hsc70 sequence. (B) Sequence alignment of bacterial, plant, and eukaryotic Hsp70s. The conserved active site cysteine is colored red; fully conserved residues are colored black, and similar side chain identities are colored gray. (C) Phosphate release kinetics of wild-type Hsc70 and C¹⁷W proteins (residues 5–554). (D) ATP-binding curve for wild-type Hsc70 and C¹⁷W (residues 5–554). The inset shows K_d values. Graphs show the mean values $\pm$ the standard deviation (SD). Data are plotted from a minimum of two biological replicates with three technical repeats each.

Figure 2. Structures of Hsc70 wild type and Hsc70 C¹⁷W bound to adenosine nucleotides. Crystal structures of Hsc70 (A) wild type bound to Mg²⁺ ·ADP and P_i, (B) C¹⁷W bound to Mg²⁺ ·ADP, and (C) C¹⁷W bound to AppNHp. (D) α -Carbon overlay depicting the high degree of similarity between the structures shown in panels A–C.

Figure 3. Hsc70 C¹⁷W structure depicting a disrupted hydrogen-bond network and altered magnesium-nucleotide coordination. (A) Hsc70 wild-type active site (gray) bound to ADP and P_i demonstrating the posthydrolysis state. The schematic diagram emphasizes the coordination of water molecules and magnesium with select active site residues. A potential potassium ion site, modeled as a water, is highlighted with a pink halo (see Discussion for details). (B) Prehydrolysis state of GRP78 (PDB entry 5F2R, orange) displaying an active site geometry analogous to that observed for wild-type Hsc70 in panel A. (C) Hsc70 C¹⁷W (slate) bound to ADP shows a loss of Mg²⁺ coordination via H₂O*. The steric hindrance of the introduced tryptophan mutation also appears to displace the H₂O* observed in wild-type Hsc70, which is absent from the C¹⁷W active site. (D) Hsc70 C¹⁷W (dark red) bound to AppNHp displaying a similarly compromised active site configuration as observed for the ADP-bound structure. Here the displaced Mg²⁺ is coordinated by D¹⁰.

Figure 4.

Hsc70 $C^{17}W$ mutation causes a significant displacement of the catalytic magnesium ion. (A) A structural overlay of 25 Hsp70 family members indicates the high degree of structural similarity.^{7,40,49,55–59,61,72–75} (B) Ligand overlays from the same Hsp70 structures shown in panel A depict the indistinguishable magnesium locations. Only two magnesium ions deviate, which belong to the $C^{17}W$ structures. (C) Quantification of the measured magnesium displacement distances; means and the SD are displayed.

Figure 5.

Thermal stability of the wild-type and Hsc70 C¹⁷W proteins in the presence and absence of magnesium and nucleotide. (A) Thermal melting data for the Hsc70 wild type in the apo, ADP-bound, or ATP-bound state in the absence (left) or presence (center) of magnesium. T_m values determined from the second derivative of the melting curves are displayed at the right. (B) Thermal melting data for Hsc70 C¹⁷W under the same experimental conditions used for the wild type; T_m values are displayed at the right. Graphs depict the mean data and SD and represent a minimum of two biological replicates with three technical repeats each. **** $p < 0.001$; $p > 0.05$ is defined as not significant (ns) by one-way analysis of variance.

Figure 6.

Difference of distance matrices between wild-type and Hsc70 C¹⁷W structures. Pairwise distance matrices for (A) Hsc70·ADP and P_i (PDB entry 6B1I) and Hsc70 C¹⁷W·ADP (PDB entry 6B1N) and (B) Hsc70·ADP and P_i (PDB entry 6B1I) and Hsc70 C¹⁷W·AppNHp (PDB entry 6B1M) were calculated and subtracted using SuperPose⁴⁴ and plotted as heat maps. Teal represents no change, and yellow or purple indicate directional distance changes of 3 Å. Distance variations between subdomains are highlighted with arrows: orange arrows for subdomains IIB and IA, pink arrows for IIB and IB, and red arrows for regions near residues 32, 191, and 214 in relation to all other domains. The insets depict the distances changes noted in the matrices mapped onto the wild-type structure. Dotted lines relate the subdomain distances (orange and pink); the α -carbon spheres (red) highlight residues that differ in distance from all other subdomains.

Figure 7.

Modeling of sulfonic acid and glutathione cysteine adducts in Hsc70. (A) Hsc70 wild-type structure from this study displaying the solvent-excluded surface of C¹⁷ and the position of the H₂O[‡]. (B) Hsc70 C¹⁷W structure bound to AppNHp showing the solvent-excluded surface of C¹⁷W and the overlapping location of the H₂O[‡] from the wild-type structure. (C) The solvent-excluded surface of sulfonic acid modeled onto C¹⁷ from the wild-type Hsc70 structure (panel A) displays a size similar to that of C¹⁷W and comes into close contact with the H₂O[‡]. (D) The solvent-excluded surface of glutathione modeled onto C¹⁷ from the wild-type Hsc70 structure (panel A) occupies a much larger space in the active site, relative to that of tryptophan or sulfonic acid. Glutathione is predicted to fully displace the H₂O[‡] and has the potential to occlude nucleotide binding.

Table 1**X-ray Data Collection and Refinement Statistics**

	Hsc70 wild type	Hsc70 C¹⁷W	Hsc70 C¹⁷W
residues	5–381	5–381	5–381
nucleotide	Mg ²⁺ ·ADP with P _i	Mg ²⁺ ·AppNHp	Mg ²⁺ ·ADP
Data Collection			
X-ray source	CHESS	CHESS	CHESS
wavelength (Å)	0.977	0.977	0.977
space group	<i>P</i> 1211	<i>P</i> 1211	<i>P</i> 1211
unit cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.8, 79.2, 76.3	73.8, 78.3, 75.9	73.4, 77.6, 75.5
α , β , γ (deg)	90, 101.5, 90	90, 101.4, 90	90, 101.2, 90
resolution (Å) ^a	47.49–2.30 (2.42–2.30)	47.37–1.90 (2.00–1.90)	47.27–1.80 (1.90–1.80)
no. of reflections			
total	157756 (21807)	282688 (31636)	344644 (42166)
unique	35296 (5561)	66389 (9353)	76551 (11025)
completeness (%)	91.9 (100.0)	99.5 (97.0)	99.3 (98.4)
multiplicity	4.5 (3.9)	4.3 (3.4)	4.5 (3.8)
<i>I</i> / σ (<i>I</i>)	12.1 (2.7)	10.7 (2.0)	8.4 (2.2)
<i>R</i> _{meas} (%)	12.7 (61.8)	14.1 (72.7)	14.8 (67.8)
<i>R</i> _{merge} (%)	11.2 (53.3)	12.3 (61.6)	13.1 (58.3)
CC _{1/2}	0.994 (0.852)	0.994 (0.702)	0.993 (0.828)
Refinement			
<i>R</i> _{work} , <i>R</i> _{free} (%)	19.1, 24.2	17.9, 23.0	18.4, 22.6
RMSD			
bond lengths (Å)	0.002	0.005	0.012
bond angles (deg)	0.47	0.742	1.19
no. of atoms			
protein	5743	5911	5858
ligands	84	76	98
water	364	854	806
average <i>B</i> factor (Å ²)			
protein (total)	26.7	19.5	20.3
ligands	28.2	18.8	24.4
water	25.3	29.2	29.6
Ramachandran (%)			
favored	97.9	98.7	98.7
outliers	0	0	0
PDB entry	6B1I	6B1M	6B1N

^aValues in parentheses are for the highest-resolution bin.