Human Hsp70 molecular chaperone binds two calcium ions within the ATPase domain

M Sriram[1], J Osipiuk[1], BC Freeman[2], RI Morimoto[2] and A Joachimiak[1,2]

Background: The 70 kDa heat shock proteins (Hsp70) are a family of molecular chaperones, which promote protein folding and participate in many cellular functions. The Hsp70 chaperones are composed of two major domains. The N-terminal ATPase domain binds to and hydrolyzes ATP, whereas the C-terminal domain is required for polypeptide binding. Cooperation of both domains is needed for protein folding. The crystal structure of bovine Hsc70 ATPase domain (bATPase) has been determined and, more recently, the crystal structure of the peptide-binding domain of a related chaperone, DnaK, in complex with peptide substrate has been obtained. The molecular chaperone activity and conformational switch are functionally linked with ATP hydrolysis. A high-resolution structure of the ATPase domain is required to provide an understanding of the mechanism of ATP hydrolysis and how it affects communication between C- and N-terminal domains.

Results: The crystal structure of the human Hsp70 ATPase domain (hATPase) has been determined and refined at 1.84 Å, using synchrotron radiation at 120K. Two calcium sites were identified: the first calcium binds within the catalytic pocket, bridging ADP and inorganic phosphate, and the second calcium is tightly coordinated on the protein surface by Glu231, Asp232 and the carbonyl of His227. Overall, the structure of hATPase is similar to bATPase. Differences between them are found in the loops, the sites of amino acid substitution and the calcium-binding sites. Human Hsp70 chaperone is phosphorylated *in vitro* in the presence of divalent ions, calcium being the most effective.

Conclusions: The structural similarity of hATPase and bATPase and the sequence similarity within the Hsp70 chaperone family suggest a universal mechanism of ATP hydrolysis among all Hsp70 molecular chaperones. Two calcium ions have been found in the hATPase structure. One corresponds to the magnesium site in bATPase and appears to be important for ATP hydrolysis and *in vitro* phosphorylation. Local changes in protein structure as a result of calcium binding may facilitate phosphorylation. A small, but significant, movement of metal ions and sidechains could position catalytically important threonine residues for phosphorylation. The second calcium site represents a new calcium-binding motif that can play a role in the stabilization of protein structure. We discuss how the information about catalytic events in the active site could be transmitted to the peptide-binding domain.

Addresses: [1]Argonne National Laboratory, Argonne, IL 60439, USA, and [2]Northwestern University, Department of Biochemistry, Molecular Biology and Cell Biology, Evanston, IL 60208, USA.

*Corresponding author.
E-mail: andrzejj@anbiw4.sbc.anl.gov

Key words: ATPase, calcium-binding motif, Hsp70, molecular chaperone

Received: 29 August 1996
Revisions requested: 26 September 1996
Revisions received: 10 January 1997
Accepted: 14 January 1997

Electronic identifier: 0969-2126-005-00403

Structure 15 March 1997, 5:403–414

Introduction

The synthesis of a large family of heat shock proteins (hsp) occurs in response to many stimuli. Under 'stress', the synthesis of cellular proteins is suppressed and only hsp proteins are made in large quantities [1–3]. A number of hsp proteins are molecular chaperones that are also essential under normal cellular conditions. Metal ions and a calcium ionophore are among the stimuli that affect the transcription of some chaperone proteins [4–7]. In addition, these proteins need metal ions for ATP binding and hydrolysis, and for chaperone activity. For example, the Hsp70 family of molecular chaperones require specific monovalent and divalent metal ions for ATP binding and hydrolysis [8–11]. ATP hydrolysis is coupled with Hsp70 molecular chaperone function, which is important in protein synthesis [12,13], protein folding [13–15], protein translocation [16–19], uncoating of clathrin coated vesicles [5], protein degradation [20] and modulation of protein expression [21,22]. Calcium-dependent autophosphorylation *in vitro* has been reported for proteins in the Hsp70 family [9,23–25], but its role *in vivo* remains unclear.

In humans, the Hsp70 chaperone family consists of four members: the stress and growth regulated Hsp70 [26]; the

abundant and constitutively expressed heat shock cognate protein Hsc70 [27] (Hsp70 and Hsc70 are present in both cytosol and nucleus); the profuse, ER-localized Grp78 (BiP) [8]; and the abundantly expressed mitochondrial MtHsp75 [28]. The synthesis of Hsp70, Grp78 and MtHsp75 proteins can be further induced in response to a specific stress [29].

Human Hsp70 chaperone is a 640 amino acid protein composed of two major domains. The 44 kDa, 388 amino acid, N-terminal domain binds and hydrolyzes ATP, whereas the C-terminal domain is required for binding peptides and folding non-native polypeptides. The C-terminal domain can be divided into two functionally relevant subdomains, an 18 kDa peptide-binding domain and a 10 kDa C-terminal domain that contains the Glu-Glu-Val-Asp (EEVD) regulatory motif [30]. It has recently been shown that the EEVD motif of the human Hsp70 molecular chaperone regulates ATP hydrolysis [30], and that it interacts with substrates and the co-chaperone, HDJ-1 [31]. Communication between Hsp70 domains appears to be an important component of Hsp70 molecular chaperone activity [30,32].

Human Hsp70 and bovine Hsc70 share sequence and function similarity. Several high-resolution crystal structures of the bovine Hsc70 ATPase domain (bATPase) including those of important point mutants, have been determined and refined [10,11,33,34]. The bATPase structure reveals a two-subdomain fold, with ATP/ADP sandwiched between the subdomains. The position of a nucleotide and three metal ions (one magnesium and two potassium) have been determined. More recently, the crystal structure of the peptide-binding domain of the chaperone DnaK in complex with peptide substrate has been determined [35]. These structures provide an initial framework for mechanistic, functional and structural analysis. Within the large family of Hsp70 chaperones there is remarkable diversity, and more structural studies are needed to understand the functions and interactions of these proteins. Here, we report the high-resolution crystal structure of the human Hsp70 ATPase domain obtained in the presence of calcium and the identification of a new calcium-binding motif.

Results and discussion

Structure determination

Crystals of a 41.9 kDa human Hsp70 ATPase domain suitable for high-resolution X-ray diffraction studies were obtained from crystallization setups containing full length Hsp70 protein, $CaCl_2$ and γ-S-ATP. Orthorhombic crystals (space group $P2_12_12_1$, unit cell parameters a = 145.47 Å, b = 63.34 Å, c = 45.95 Å, $\alpha = \beta = \gamma = 90°$) diffract X-rays to at least 1.3 Å resolution using synchrotron radiation. Structure determination was carried out by molecular replacement using X-PLOR version 3.1 [36] and bATPase (obtained from the Brookhaven Protein Data Bank, PDB code 1atr) was used as a starting model. Data statistics are shown in Table 1. Freezing of the crystal to 120K caused the unit cell dimensions to contract, but contractions were not uniform in all directions. The largest change, 2.6% (3 Å) of the unit cell, was observed along the b axis, an intermediate change was seen along the c axis (2.1%, 1 Å), with almost no change observed along the a axis (0.1%). The changes observed by Wilbanks *et al.* [11] upon freezing of the bATPase are more uniformly distributed: 2% along the b axis, 1.3% along the a axis and 1.5% along the c axis. This anisotropy may reflect sequence differences that affect the interaction of protein molecules in the crystal or a difference in cryoprotectant and freezing procedure.

The quality of the electron-density map was very high (Fig. 1), as a result of high resolution and the completeness of data. This allowed the determination of sidechain positions (e.g. Lys77, Tyr137 and Arg264) that could not be determined in the 1.70 Å bovine structure (PDB code 1hpm). The hATPase protein sequence mutations (as derived from the sequence of the gene) in well ordered regions of the protein were found directly from the X-ray data. Isoleucine was identified at position 7 instead of valine. This substitution was confirmed by the direct sequencing of protein from a crystal. Alignment of Hsp70 sequences shows that this position is interchangeably valine or isoleucine. An identical valine to isoleucine substitution was also found at position 18. In the Hsp70 family, valine is highly conserved in this position, where phenylalanine, leucine or isoleucine are infrequently found. The mutant protein used in this study showed virtually identical ATPase and protein-folding activity to a previous Hsp70 preparation (our unpublished observations). The relevance of these substitutions to the structure will be discussed later. The Ramachandran plot confirms the high quality of the structure and shows that 91.4% of non-glycine residues are in most favored regions with the remaining residues in the preferred regions. The averaged isotropic temperature factor for all protein atoms is 17.1 $Å^2$. This is significantly lower than that (32.6 $Å^2$) determined for the room temperature structure of bATPase (PDB code 3hsc) [11,33] and is closer to that of bATPase structure determined at 100K (13.3 $Å^2$; PDB code 1hpm) [10]. Data collection at 120K clearly lowered the dynamic disorder in the crystal; however, static disorder remains a factor, because several sidechains in loop regions are less well defined in hATPase (regions 22–24, 188–190 and 213–215 and residue 250). The products of ATP hydrolysis, ADP and inorganic phosphate (Pi), show a very low isotropic temperature factor of 9.4 $Å^2$. All amino acid sidechains in the nucleotide-binding pocket also show very little local disorder suggesting that the ligand has a 'crystallization' role on the protein, as previously observed [10]. This is also applicable to the metal ions and solvent molecules in the catalytic site.

Table 1

Statistics of data collection and restrained refinement using X-PLOR for the native crystal of hATPase*.

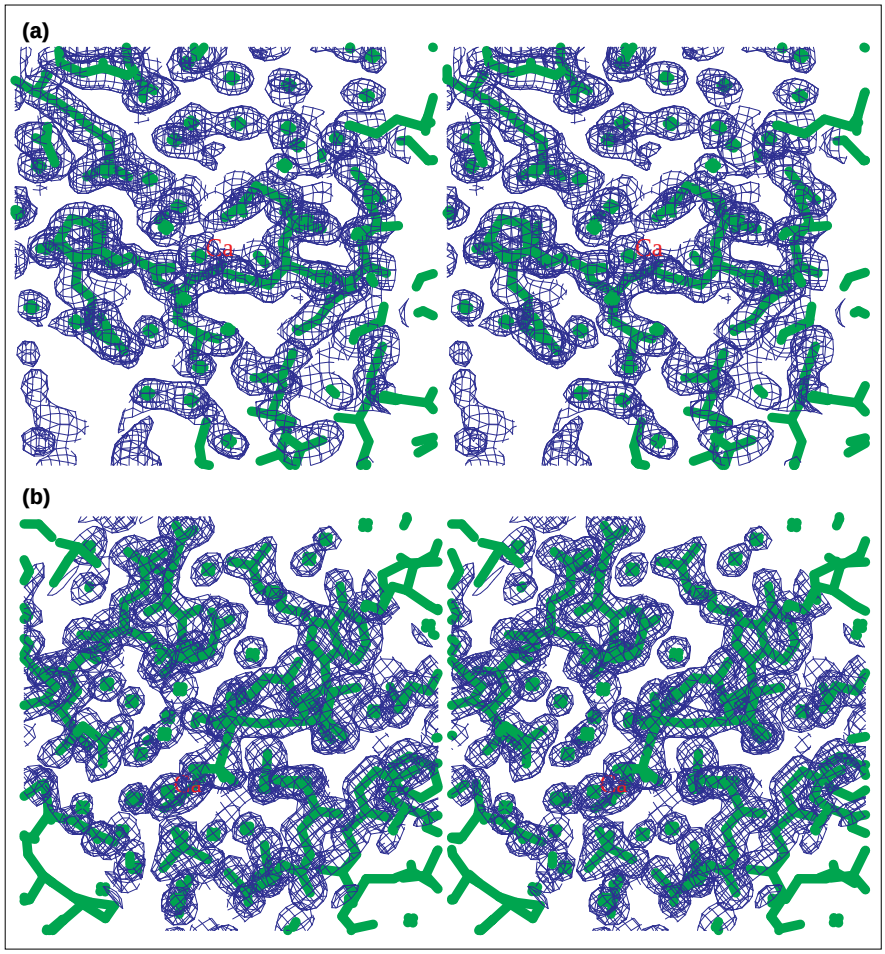
Resolution shells (Å)	> 3.67	3.03	2.69	2.46	2.30	2.17	2.06	1.98	1.90	1.84	overall
Number of reflections	3426	3522	3600	3591	3554	3419	3533	3552	3492	3392	35 081
Coverage (%)	89.4	94.6	97.6	97.6	97.5	93.3	97.6	97.7	96.2	94.2	95.5
(I/σ)	34.3	34.2	36.1	32.0	30.4	25.5	20.8	17.8	12.0	9.6	28.3
R_{sym} (%)†	2.8	3.2	3.7	5.0	4.7	7.8	9.1	10.8	18.1	20.4	4.9
Working R factor (F > 2σF)‡	16.2	15.8	18.3	19.9	21.2	24.3	23.4	25.4	29.2	29.1	20.2
Free R factor§	19.2	19.5	18.1	20.5	21.4	25.6	26.0	25.8	31.7	27.2	21.9

*Coverage (%), I/σ, R_{sym}, R factor, and free R factor are shown for 6.0 to 1.84 Å resolution shells. †$R_{sym} = \Sigma < |I(h) - I(h')| > / \Sigma I(h')$, where $< |I(h) - I(h')| >$ represents the average of the absolute deviation of a reflection I(h′) from the average I(h) of its symmetry and Freidel equivalents. ‡R factor = Σ|Fobs| – |Fcalc| / Σ|Fobs|. §Free R factor was calculated with a random 10% (T) of the intensities evenly distributed throughout the resolution range, working R factor was calculated with the remaining intensities. Final model, including residues 3–382 and 415 water molecules, has bond and angle rms deviations of 0.006 Å and 1.26°.

Structure of the hATPase domain

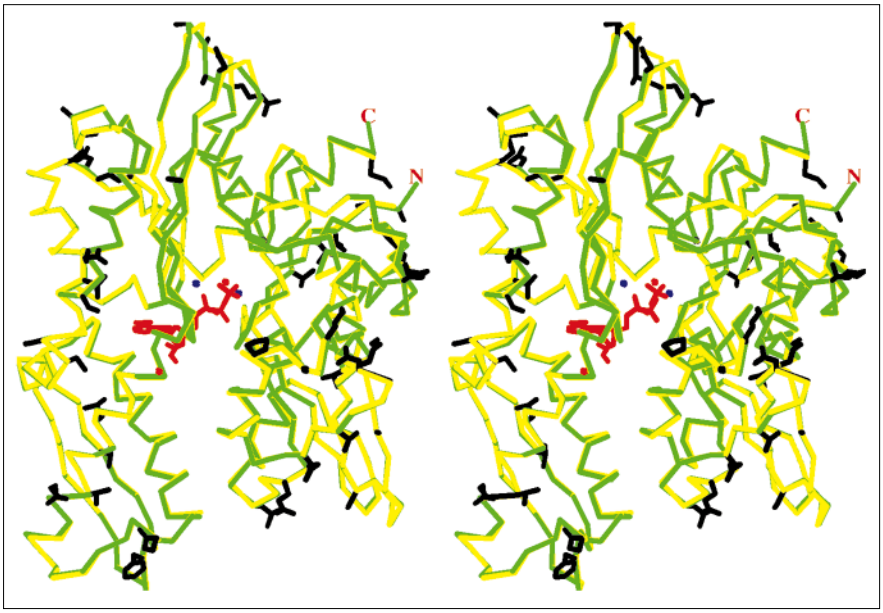
This is the first reported high-resolution crystal structure of the hATPase domain. The structure is very similar to the constitutively expressed bATPase homologue [10,11,33]. The sequence of hATPase (residues 3–382) differs from bATPase at 45 residues that are distributed throughout the sequence, but these changes are localized almost exclusively on the protein surface (Fig. 2). Differences between the hATPase and bATPase domains are distributed on two surfaces: 16 substitutions occur at the lower tips of the two sub-domains (residues 2–190 and 191–383), which are separated by the cleft, and 10 substitutions

Figure 1



Stereo view of $2F_o–F_c$ electron-density maps calculated using model phases. (a) Surface calcium-binding site countered at 1σ level, the calcium ion is labeled ‘Ca’ in red. (b) Catalytic site countered at 1σ level, showing the ADP, Pi, and relevant amino acid residues.

Figure 2



Comparison of human Hsp70 ATPase (hATPase) with bovine Hsc70 ATPase (bATPase). Stereo view of least-square fit of human (green) and bovine (yellow) ATPases. Sidechains of amino acid residues that differ in hATPase from those in bATPase are shown in black. ADP and Pi are shown in red. Calcium and sodium ions are shown as dots, red and blue, respectively.

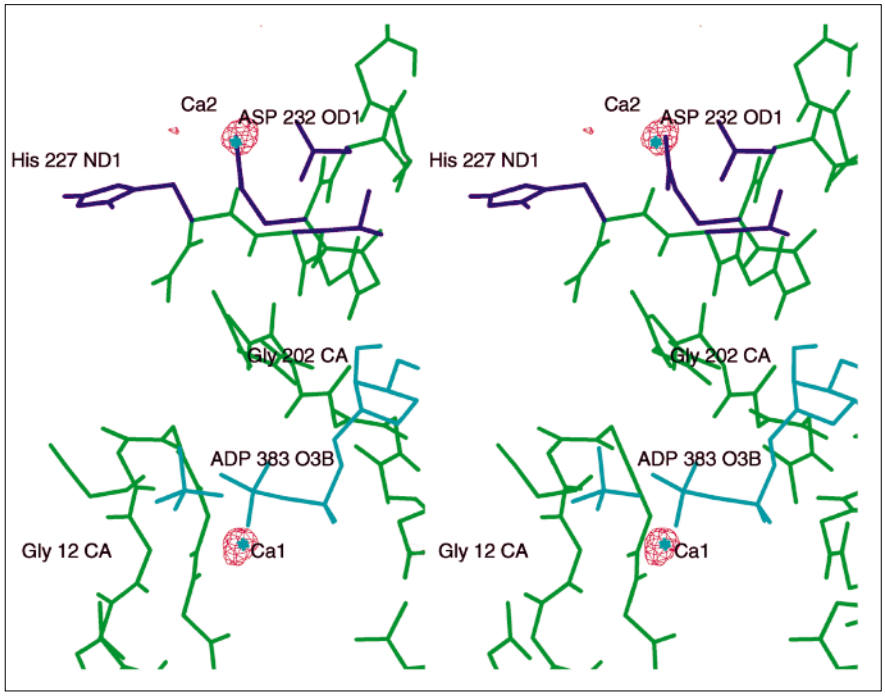
cluster on the surface near the N and C termini (Fig. 2). In the structure of hATPase, two additional amino acids are clearly visible in electron density on the N and C termini. These amino acids protrude directly into solvent and are only ~11 Å apart. In hATPase, N-terminal residues Ala2 and Lys3 (determined by amino acid sequencing) can extend away from the ATPase surface and can contact the peptide-binding domain. These residues are part of a β strand that interacts directly with the inorganic phosphate through Thr13 and Thr14 (see below). The C-terminal α helix crosses over the β strand and contacts the β phosphate group of ADP through Asp366. Such an arrangement can potentially detect a shift in the relative position of β and γ phosphates of ATP during its hydrolysis, and suggests a possible way of transmitting information about γ phosphate movement to the peptide-binding domain. This hypothesis is reinforced by the observation of Zhu *et al.* [35] that the N-terminal fragment of the peptide-binding domain (residues 389–394) can assume two distinctly different conformations in the crystal. These conformational states may be relevant to the ATP-dependent conformational change and the function of latch mechanisms in peptide binding and release [35].

Two mutations Val7→Ile and Val18→Ile, identified in the structure, have very little effect on the local structure of hATPase as compared with bATPase that has valine in both sites. Ile7 and Ile18 are part of a hydrophobic core near the N terminus. The δ methyl group of Ile7 points into a hydrophobic cavity and makes contact with Ile9 and Val139. Very small changes in the position of Val139 could be in part attributed to the Val7→Ile substitution. Ile18 is in the vicinity of Ile7 and its δ methyl group makes contact with Ile29, Ala131 and carbonyl of Gly19. These two substitutions may increase stability of this hydrophobic region.

The high-resolution crystal structures of hATPase (1.84 Å) and bATPase (1.70 Å, PDB code 1hpm) containing ADP and Pi were least square fitted and show a 0.36 Å root mean square (rms) deviation for all mainchain atoms. As expected, the largest differences are observed in the loop regions and in the regions of amino acid substitutions. The interior of both proteins is conserved in both sequence and structure, although some sidechains refined with different conformations. There is a high degree of structural similarity of both proteins consistent with a universal mechanism of ATP hydrolysis among all Hsp/Hsc70 molecular chaperones [11]. The crystal structures of human and bovine ATPase domains with bound ligand show remarkably well ordered proteins. The ATP, ADP+Pi and ADP bound states are virtually identical ([10]; our unpublished observations). It was shown recently for the GroEL chaperonin that minimal changes in the structure alone can accommodate ATP [37,38]. Very small differences between ADP and ATP bound states do not correlate well with the suggested large conformation change upon peptide binding and the proposed dynamic nature of protein folding. We suggest that the transition from one state to the other is important for the protein conformational switch that facilitates protein folding. In addition to protein oligomerization, conformational reorganization of domains due to peptide binding or involvement of other protein cofactors may be required for the

Figure 3

Stereo view of a anomalous difference Fourier map showing position of the calcium ions, as calculated with the data between 6.0 and 1.84 Å, using model structure factors and phases (but excluding contribution of calcium ions). The only significant features in this map correspond to positions of calcium ions. Map is contoured at 3σ level and calcium ions are labeled as blue stars. Both ADP-bound (Ca1) and loop 229–232-bound (Ca2) calcium ions are shown in this view in red. Protein is shown in green, ADP and free phosphate in blue and conserved sidechains of calcium-binding loops in purple.



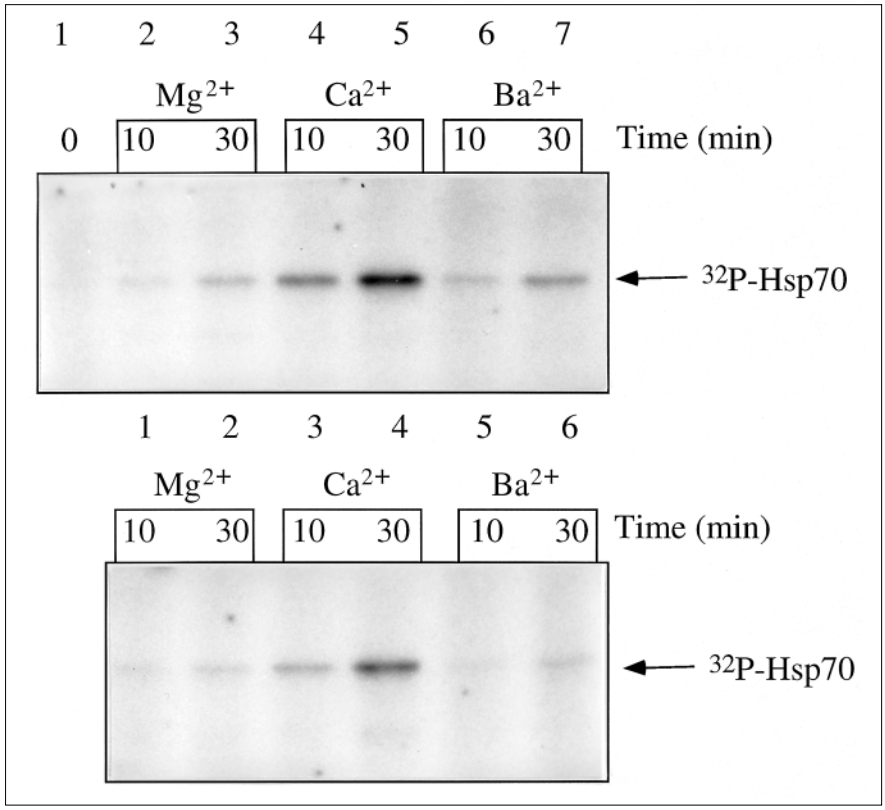
proposed allosteric long-range conformational changes to occur [32].

This structure of the hATPase domain reveals 415 solvent molecules (13 water molecules less than were found in the bATPase (PDB code 1hpm)). On average, one water molecule was found per amino acid residue. Many of the solvent molecules share the same positions in both structures, although a number of water molecules appear to occupy different positions in hATPase. The averaged isotropic temperature factor for all solvent molecules is 34.7 Å^2. The solvent forms an extensive network on the surface of the protein that may contribute to its stability. The cleft between the sub-domains contains a large number of well ordered solvent molecules. The solvent structure appears to be disrupted by hydrophobic side chains located on the surface near N and C terminus of hATPase; this suggests possible contact points that the hATPase domain may have with the C-terminal domain.

The catalytic site

ADP is bound in the cleft between two sub-domains of hATPase and is located within the protein body except for the edge of adenine that is solvent exposed (Fig. 1b). The adenine base is sandwiched between the hydrophobic segments of two arginine residues, Arg272 and Arg342; this arrangement is similar to the binding of L-Trp to *trp* repressor [39]. The arginine guanidinium group stabilizes the solvent molecules, which are linked by hydrogen bonds to N7 and N6 of the adenine base. Such an ADP-binding motif has been described earlier for bATPase [33]; a similar motif is found in actin that shares very little sequence identity but high structural similarity with the ATPase domain described here [40]. In the ADP-binding site of hATPase, the ribose hydrogen bonds to Asp268 and Lys271 and the α and β phosphates project into a protein cavity that contains metal ions and a number of well ordered water molecules. The β phosphate is rotated by 40° with respect to its position in the bovine homologue in complex with magnesium and potassium ions (PDB code 1hpm). A similar rotation of the β phosphate has been found in the crystal structure of bATPase containing magnesium and sodium ions [10]. Flaherty *et al.* [10] attributed this rotation to the presence of sodium ions. The structure of hATPase contains calcium and sodium ions, confirming that the observed rotation of the β phosphate is due to the presence of sodium ions. A clear electron density for the Pi group was located 4.8 Å from the β phosphate of the ADP shifted, with respect to its position in bATPase, by 0.39 Å in the direction of Thr204 (Fig. 3). The Pi group is coordinated by a salt bridge with Lys71, hydrogen bonds to Thr13 and Thr204 and it interacts directly with a calcium ion (Fig. 1b). A water molecule mediates additional interactions with the protein mainchain at positions 202, 203 and 204. The Pi-binding site is on the protein face opposite the highly conserved Gly32 loop that has been implicated in binding of nucleotide release factor (GrpE) to the ATPase domain of DnaK [41]. There are

Figure 4



In vitro phosphorylation of human Hsp70. Reactions of 20 μl, containing 50 mM buffer, 50 mM KCl, 1 mM DTT, 5 μM ATP, 0.1 μCi of ^{32}P-γ-ATP, 10% glycerol, 30 pmol Hsp70 and 1 mM of divalent metal, were incubated at 37°C. The reaction was stopped by addition of 2% sodium lauryl sulfate, samples were loaded onto a 12% polyacrylamide gel and run under denaturing conditions. Gels were dried and autoradiographed. The top panel shows reactions in the presence of MES buffer pH 6.2 and the bottom panel shows reactions in imidazole buffer pH 7.0.

potential channels for Pi exit to the protein surface. Release of the inorganic phosphate group has been implicated in the conformational transition of Hsp70 molecular chaperone [42].

The calcium ion and two sodium ions were initially located in the catalytic site by analogy with the structure of bATPase [10,33]. An additional calcium ion was found on the surface of protein and will be discussed later. The two calcium sites represent the fourth and the fifth highest peaks in the $2F_o–F_c$ map (three phosphate groups being the highest). To confirm the positions of calcium ions in the structure, we have calculated the anomalous difference Fourier map (Fig. 3). This map shows only two major peaks, corresponding to two calcium ions. The calcium ion bound to the active site of hATPase is shifted 0.8 Å from the magnesium ion position in bATPase. Sodium ions moved in the same direction as calcium, 0.4 Å from the respective position of potassium ions in bATPase. The coordination of calcium ion in hATPase is similar but not identical to the magnesium coordination in bATPase containing ADP and Pi (PDB code 1hpm), and it is quite different from the calcium coordination in bATPase in complex with the ATP analogue, AMP-PNP (PDB code 1ngi) [10]. In the bATPase structure, the magnesium ion is coordinated by the oxygen of β phosphate, the oxygen of Pi and four water molecules. In the hATPase structure, calcium is in very similar position, octahedrally coordinated by the oxygen of β phosphate (at a distance of 2.41 Å), two oxygen atoms of Pi (2.58 Å and 2.84 Å, respectively) and four water molecules — H_2O415 (2.54 Å), H_2O565 (2.56 Å), H_2O657 (2.66 Å) and H_2O692 (2.68 Å). This coordination scheme may explain the observed rotation and shift of the γ phosphate. As discussed earlier, the β phosphate rotation is consistent with the presence of sodium ions. In contrast, coordination of calcium in bATPase in complex with AMP-PNP involves a closer interaction with the carboxylates of Asp199 and Glu175 than the oxygen atoms of β and γ phosphates [10]. Actin, which shows structural homology to the ATPase domain, also contains calcium in the active site [43]. In the structure of the ATP–actin complex, calcium is bound to the β phosphate and γ phosphate oxygens at distances similar to those observed for hATPase. In the ADP–actin complex, calcium is bound to β phosphate oxygen. Two aspartic acids and a glutamine residue have an indirect role in coordinating the calcium ion in actin [43]. The binding mode of calcium in the ADP/Pi–hATPase complex appears most similar to the binding of magnesium in ADP/Pi–bATPase. This observation is consistent with other data suggesting that calcium and magnesium ions often bind to the same sites in proteins with similar coordination geometry [44].

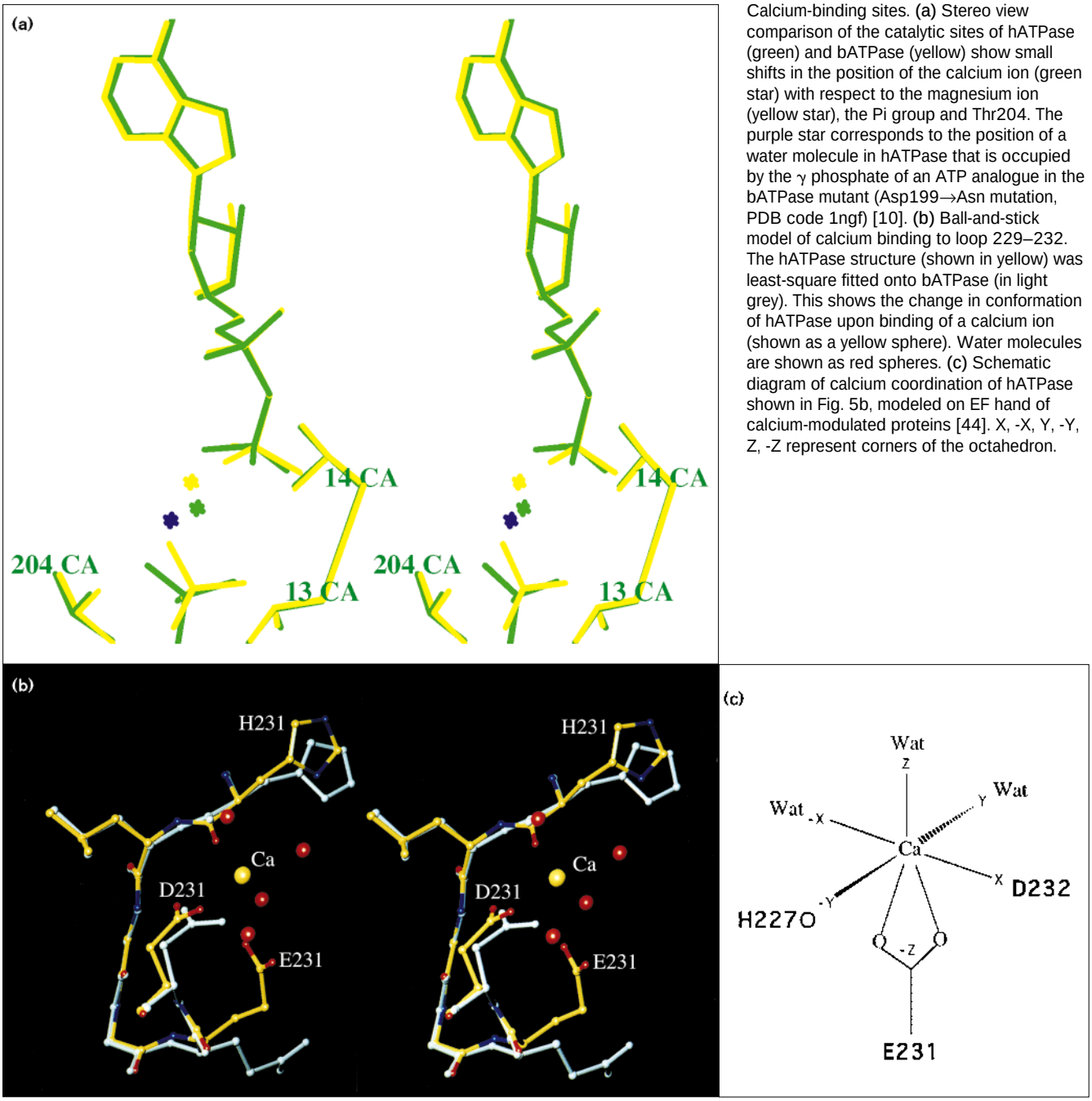
In the ATPase active site, the metal ions position and neutralize the negative charge of the phosphate group during catalysis. Mutagenesis studies combined with X-ray crystallographic analysis of active-site residues suggest that conserved Lys71 is a catalytically essential residue that affects ATP hydrolysis [45]. The proposed mechanism of ATP hydrolysis suggests the role of Lys71 in accepting a proton from the hydroxide ion or water molecule involved in in-line nucleophilic attack [10,11,25,45]. Phospho-threonine was postulated as an intermediate of ATP hydrolysis, but mutational studies suggest that phospho-threonine may not be an obligatory intermediate [23–25]. We analyzed the high-resolution structure to evaluate potential intermediates of ATP hydrolysis. ATP is also hydrolyzed in the presence of calcium ions ([23,24]; our unpublished observations). In the presence of calcium the formation of phospho-threonine was detected for two members of Hsp70 family — DnaK and BiP [9,23–25]. We tested whether human Hsp70 can be phosphorylated *in vitro* in the presence of divalent metal ions. Figure 4 shows that all tested metal ions (Mg^{2+}, Ca^{2+} and Ba^{2+}) support phosphorylation of human Hsp70 to some extent, calcium being the most effective. Phosphorylation is also observed under conditions similar to those used in crystallization experiments. Magnesium ions inhibit phosphorylation of BiP [23,24], but in the presence of Mg^{2+} low level phosphorylation is seen for human Hsp70 chaperone (Fig. 4). However, it is important to point out that even in the presence of calcium the level of phosphorylation is relatively low. This agrees with an earlier observation of Zylicz *et al.* [9] that only approximately 10% of DnaK is phosphorylated. By analogy with DnaK and BiP, we assume that in the presence of divalent metal ions the activated γ phosphate can be transferred to Thr204. This structurally conserved residue was suggested as a phosphate acceptor [23–25]. However, point mutations of Thr199 (equivalent to Thr204 in DnaK), significantly lowered, but did not completely abolish, ATPase activity [25]. This suggests that an alternative sidechain can serve as a phosphate acceptor or that the phospho-threonine is not an obligatory intermediate in ATP hydrolysis.

The observed phosphorylation of the human Hsp70 in the presence of calcium suggests that the structure of hATPase (+ Ca^{2+}) corresponds to the state in which *in vitro* phosphorylation is more favored. Small changes in the ATPase structure caused by binding of a calcium ion presumably alter the mechanism of ATP hydrolysis or stabilize the phospho-threonine intermediate. Our data suggest that these structural changes may be attributed to differences in the ionic radii of the metal ions, with calcium (0.99 Å) ion being larger than magnesium (0.65 Å) and smaller than barium (1.35 Å). Analysis of the hATPase catalytic site containing a bound calcium ion provides a possible explanation for the mechanistic difference. A concerted shift of metal ions and protein sidechains is observed in the direction away from the β phosphate of ADP and towards Thr204 (Fig. 5a). During catalysis, it is likely that the shift to a new position of hydrolyzed phosphate, coordinated by calcium, occurs more rapidly than the relaxation of protein structure. This would temporarily bring the activated phosphate in closer proximity to Thr204, allowing the phosphate to be transferred to Thr204 more efficiently in the presence of the larger calcium ion. Hence, the question arises whether phospho-threonine is formed during ATP hydrolysis. The structure of human ATPase shows that the distance between the Pi-phosphorus atom and the Thr204 γ oxygen is 3.50 Å, and the distance between the phosphate oxygen and the Thr204 γ oxygen is 2.60 Å. This threonine residue is in position for in-line nucleophilic attack on the γ phosphate and formation of phospho-threonine. However, mutation of Thr204 does not abolish ATPase activity completely [46]. Inspection of the positions of all relevant atoms in the catalytic site of hATPase reveals that Thr13 appears to be structurally equivalent to Thr204, so that both threonine residues could be involved in phosphorylation and ATP hydrolysis (Fig. 5a). Analogous distances measured from the Pi-phosphorus atom and the phosphate oxygen to the Thr13 γ oxygen are 3.58 Å and 2.61 Å, respectively. Therefore, Thr13 is in position for in-line nucleophilic attack on the γ phosphate. This may explain why a single mutation of either of the threonine residues does not completely abolish ATP hydrolysis. Threonine residues 13 and 204 are structurally conserved among Hsp70 chaperones. However, mutational studies suggest certain asymmetry in the properties of Thr13 and Thr204. Mutation of Thr204 more strongly affects ATP hydrolysis than mutation of Thr13, and Thr204 is a primary acceptor of the activated phosphate during *in vitro* phosphorylation [25]. Our *in vitro* phosphorylation data suggest that during ATP hydrolysis, the protein is phosphorylated in the presence of divalent ions, but phosphorylation is about 3–5 times faster in the presence of calcium than in the presence of magnesium or barium ions. This is consistent with the role of divalent ion in positioning of the phosphate group for phosphorylation. In addition, proximity of two threonine residues could result in hydrolysis of a phospho-threonine intermediate (directly or through activated water molecule). More studies are needed to define the role Lys71, Thr204, Thr13 and metal ions in ATP hydrolysis and phosphorylation.

The second calcium-binding site

A new calcium-binding site on Hsp70 has been determined from the electron density of hATPase. This high occupancy calcium site was identified (at crystallization conditions of 20 mM $CaCl_2$) in four ways: firstly, its high electron density; secondly, by a strong peak in the anomalous map (Fig. 3); thirdly, by its reasonable temperature factor (22.7 $Å^2$); and lastly, by its stereochemical similarity to calcium sites found in other calcium-modulated proteins (Fig. 5b,c).

Figure 5

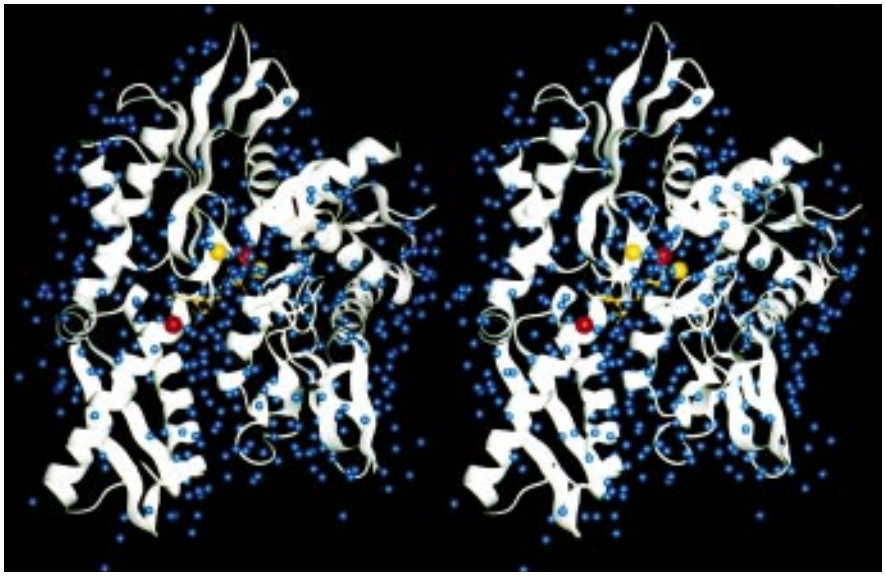


Calcium-binding sites. **(a)** Stereo view comparison of the catalytic sites of hATPase (green) and bATPase (yellow) show small shifts in the position of the calcium ion (green star) with respect to the magnesium ion (yellow star), the Pi group and Thr204. The purple star corresponds to the position of a water molecule in hATPase that is occupied by the γ phosphate of an ATP analogue in the bATPase mutant (Asp199→Asn mutation, PDB code 1ngf) [10]. **(b)** Ball-and-stick model of calcium binding to loop 229–232. The hATPase structure (shown in yellow) was least-square fitted onto bATPase (in light grey). This shows the change in conformation of hATPase upon binding of a calcium ion (shown as a yellow sphere). Water molecules are shown as red spheres. **(c)** Schematic diagram of calcium coordination of hATPase shown in Fig. 5b, modeled on EF hand of calcium-modulated proteins [44]. X, -X, Y, -Y, Z, -Z represent corners of the octahedron.

The calcium metal ion is bound within a well defined pocket (Figs 1,3,5 and 6). Salt bridges were formed between the calcium ion and Glu231 (X) (2.47 Å and 2.79 Å) and Asp232 (-Z) (2.89 Å and 3.36 Å). Calcium is also bound to the mainchain carbonyl of His227 (-Y) (2.38 Å), stabilizing a loop on the surface of the protein. In addition, calcium is coordinated by three water molecules — H_2O803 (Z, 2.69 Å), H_2O656 (-X, 2.62 Å) and H_2O549 (Y, 3.99 Å). This new metal-binding motif is formed at the junction between a β sheet (190–225) and α helix (230–250) and is in close proximity to the catalytic site. The nearest distance between the calcium ion and ADP β phosphate oxygen is 12.24 Å, and between Ca^{2+} and ribose 3′O is 10.72 Å. The Pi is 11.54 Å from the calcium ion. Although the separation of calcium from nucleotide seems large, the interaction is mediated by an oriented loop. Carbonyls 202 and 203 of the glycine-rich loop point out toward the positively charged calcium ion. Carbonyl

Figure 6

Stereo view of the hATPase structure (white ribbon), showing ADP (yellow), sodium ions (yellow spheres), calcium ions (red spheres) and water molecules (blue spheres) bound to the protein.



202 forms a direct hydrogen bond with the amide of the calcium-coordinating Glu231 and carbonyl 203 forms a water mediated interaction with calcium. The amide groups of residues 202, 203 and 204 point out toward phosphate groups (Pi and β phosphate) of ADP. All three amide groups are in position to form a hydrogen bond with a nested water molecule that bridges the β phosphate of ADP with the Pi group.

This water molecule is also found in the structure of the ADP-containing bATPase [10]. In the structures of ATP-containing bATPase Asp199→Asn mutant and AMP-PNP-containing bATPase, the γ phosphate occupies the position of this nested water and interacts directly with glycine-rich loop [10]. Although the amino acid sequence of this loop is identical and the structure of the mainchain is very similar to those of hATPase, bATPase does not contain a metal ion at this site, because the Glu231 sidechain is oriented very differently (Fig. 5b). In fact, Glu231 makes a water-mediated contact with Asp69. Unexpectedly, the structure of calcium-containing bATPase also does not contain a metal ion at this site [10], perhaps because the crystallization was done in high pH. The binding of calcium ion causes rearrangement of several sidechains in its vicinity (His227, Glu231, Asp232 and Asn235) and may affect the interaction of Hsp70 with other cofactors. Calcium most likely will contribute to stability of this region. It is also possible that other metal ions could bind to this site. Several calcium-binding sites were found in actin. The primary calcium-binding site is important in ATP hydrolysis and the secondary sites have roles in the polymerization of actin [43]. The sequence of the loop that coordinates calcium ion in hATPase His-Leu-Gly-Gly-Glu-Asp is completely conserved among Hsp70, Hsc70 and Grp78. It is also found in adenylate cyclase [47]. Grp78 and adenylate cyclase have been shown to be regulated by calcium [5–7,48]. The biological significance of this metal-binding site in the ATPase domains of heat shock proteins remains to be seen.

We have shown here, using high-resolution X-ray crystallography, that the structure of Hsp70 hATPase is similar to Hsc70 bATPase. We have found two calcium ion binding sites within the ATPase domain. One calcium binds to β phosphate of ADP and Pi, suggesting a possible role of the metal ion in *in vitro* phosphorylation of Hsp70. The other is a new generic calcium-binding site, which is on the surface of the protein and at present, has no known function. These results call for more detailed functional analysis.

Biological implications

The 70 kDa heat shock protein (Hsp70) is a major molecular chaperone in eukaryotes, including humans. Hsp70 participates in protein synthesis, protein folding, protein translocation, uncoating of clathrin coated vesicles, protein degradation and modulation of protein expression. The molecular chaperone activity is functionally linked with ATP hydrolysis and phosphate release. In addition, Hsp70 proteins require metal ions for ATP binding and hydrolysis, and for chaperone activity. The ADP-bound state of Hsp70 shows higher affinity for polypeptides than its ATP-bound state. ATP binding and hydrolysis by Hsp70 affects the relative conformation of its domains and its interactions with other protein cofactors, thus affecting the chaperone function of Hsp70. Human Hsp70 has two major domains: its N-terminal ATPase domain

binds and hydrolyses ATP; the C-terminal domain is required for polypep-tide binding and folding. Communication between the Hsp70 domains appears to be important for its chaperone activity.

The first crystal structure of the human Hsp70 ATPase (hATPase) domain determined at 1.84 Å reported here is similar to its constitutively expressed (Hsc70) bovine homologue, bATPase. Differences are found in the loops, the sites of amino acid substitution and the metal-binding sites. The structural similarity of hATPase to bATPase and the sequence similarity within the Hsp70 family suggest that the mechanism of ATP hydrolysis is universal among all Hsp70 proteins. The mechanism of ATP hydrolysis has been studied by point mutations and X-ray crystallography of relevant mutant proteins. These studies have determined catalytically critical amino acid residues and have shown unexpectedly small differences between the ADP-, ADP+Pi- and ATP-bound states. The transmission of information from the active site to the peptide-binding domain appears to be critical for peptide binding and release. We suggest that the transition from one state to the other is important for the protein conformational change that facilitates protein folding. We identified residues (Thr13, Thr14 and Asp366) that are likely candidates for transmitting the information about catalytic events in the active site to the peptide-binding domain.

Unexpectedly, two calcium sites were identified in the crystal structure of hATPase. The first calcium binds within the catalytic pocket, bridging ADP and inorganic phosphate, in a position that corresponds to the magnesium-occupied site in the bATPase. The second calcium, which is not found in bATPase, is tightly coordinated on the hATPase protein surface by Glu231, Asp232 and carbonyl of His227. This new metal-binding motif is formed at the junction between a **β** sheet (residues 190–225) and **α** helix (230–250) and is in close proximity to the catalytic site. Like other chaperones of the Hsp70 family, human Hsp70 is phosphorylated *in vitro* in the presence of calcium. The calcium-bound structure of hATPase represents a state in which phosphorylation can occur and is therefore relevant to understanding the mechanism of ATP hydrolysis. From the crystal structure of hATPase, we propose that protein sidechains in the catalytic site, in particular threonine sidechains, can serve as an acceptor of the phosphate group during ATP hydrolysis. Our structure also provides detailed information about small but important movements of ions and sidechains. These data are consistent with earlier mutational analysis. Potentially, phosphorylation in the presence of calcium could serve as a regulatory function, because at high calcium concentrations a fraction of the Hsp70 chaperone molecules could become phosphorylated and thereby arrested in one state or inactive.

Materials and methods

Protein purification

Protein extracts from IPTG-induced cells were prepared at 4°C, and the recombinant proteins were purified by a combination of anion exchange and affinity chromatography [30]. The crude extract was loaded onto a 200 ml DEAE fast-flow Sepharose column (Pharmacia-LKB) and eluted with a 50–350 mM NaCl gradient over five column volumes. The fractions, containing Hsp70 protein, were pooled and recirculated over a 20 ml ATP-agarose column (Sigma; C-8 linkage), washed with 2.0 M NaCl equilibrated to 50 mM NaCl and eluted with 10 ml of column buffer, containing 10% glycerol and 50 mM Mg-ATP. The Hsp70-containing fractions were pooled, concentrated by ultrafiltration in a Centriprep-10 (Amicon), desalted over a G-25 column and dialyzed for 4 h in 20 mM Tris (pH 6.9, at 4°C), 0.1 mM EDTA and 100 mM NaCl. The protein sample was then loaded onto a 6 ml ResourceQ column (Pharmacia-LKB) and eluted with a 50–400 mM NaCl gradient over five column volumes. The fractions containing Hsp70 protein were pooled, concentrated by ultrafiltration in a Centricon-10 (Amicon) and dialyzed for 3 days against 20 mM Tris, pH 6.9, 0.1 mM EDTA and 100 mM NaCl at 4°C. Following dialysis, the protein concentrations were determined as an average of three measurements utilizing the BCA assay (Pierce) relative to a standard solution of bovine serum albumin (BSA).

Crystallization

Full length Hsp70 protein at 16 mg ml^{-1} was mixed with equal volume of reservoir, containing 25 mM imidazole buffer pH 7.0, 8% PEG 8000, 20 mM $CaCl_2$ and 1 mM γ-S-ATP in hanging drop set-ups. Protein crystals suitable for X-ray diffraction studies typically grow in four weeks at 10°C. The crystals contained a 41.9 kDa proteolytic fragment consistent with residues 2–382 of human Hsp70. The size of the polypeptide was determined by mass spectroscopy and N-terminal sequencing of the protein from the crystal (the WM Keck Foundation, Biotechnology Resource Laboratory, Yale University). Crystals belong to orthorhombic space group $P2_12_12_1$ with unit cell parameters a = 145.47 Å, b = 63.34 Å, c = 45.95 Å, $\alpha = \beta = \gamma = 90°$, and diffract X-rays to at least 1.3 Å resolution using synchrotron radiation. The solvent volume fraction in the crystal is 45%.

In vitro *phosphorylation of human Hsp70*

The phosphorylation reaction was carried essentially as described by Zylicz *et al.* [9] for DnaK. Samples of 20 μl containing 50 mM MES buffer pH 6.2, 50 mM KCl, 1 mM DTT, 5 μM ATP, 0.1 μCi of ^{32}P-γ-ATP, 10% glycerol, 30 pmol Hsp70 and 1 mM of $MgCl_2$, $CaCl_2$ or $BaCl_2$ were incubated at 37°C. Phosphorylation was also evaluated under buffer conditions used in crystallization experiments (imidazole buffer pH 7.0). The reaction was stopped by addition of 2% sodium lauryl sulfate, and samples were loaded onto a 12% polyacrylamide gel run under denaturing conditions. Gels were dried and autoradiographed.

Data collection and processing

The protein crystals were stabilized in hanging drops with 10% PEG 8000 at 4°C for one week and then glycerol was added at 20% of final concentration over a period of 4 h. Crystals were washed with cryoprotecting solvent and flash-frozen in liquid propane at liquid-nitrogen temperature. Frozen crystals can be stored in liquid nitrogen for extended periods of time. The complete data set was collected at 120K from a single crystal at Brookhaven National Laboratory NSLS X25 beam line to 1.84 Å using MAR-area detector. Images were processed with DENZO and scaled with SCALEPACK [49]. Data collection at 120K significantly reduced X-ray damage to the crystal.

Structure determination and refinement

Structure determination was carried out by molecular replacement using X-PLOR, version 3.1 [36] and bATPase (obtained from the Brookhaven Protein Data Bank, (PDB code 1atr)) was used as a starting model. Structure was refined as a rigid body with program X-PLOR. Data from 6.0 to 4.0 Å ($F > 2\sigma F$) were used in this stage, yielding an R factor of 34%. One cycle of simulated annealing with data from 5 to 2.5 Å ($F > 2\sigma F$) reduced the R factor to 25.9%. The electron-density

map was calculated and the model was adjusted manually in several cycles, using $2F_o–F_c$ and $F_o–F_c$ maps. Manual adjustments of the model and inspection of waters located by ARP [50] were performed on SGI Extreme color graphic workstation using version 5.10.3 of program O [51]. The structure was further refined using the simulated annealing slow-cooling protocol with the program X-PLOR. Final refinement included all data between 6 and 1.84 Å (35 081 independent reflections to 2σ) and 3382 atoms. The final structure yielded crystallographic R factor 20.3% and free R factor 21.9% with excellent stereochemistry (bond rms deviation is 0.006 Å, and angle rms deviation is 1.26°, Table 1).

Accession numbers

Atomic coordinates for the human Hsp70 structures have been deposited with the Brookhaven Protein Data Bank.

Acknowledgements

We thank L Berman, M Capel and R-g Zhang for help with the data collection at NSLS X25 beam line, and W Minor and Z Otwinowski for providing a copy of DENZO and SCALEPACK. This project was supported by the US Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-Eng-38. BGF was supported by a NIH predoctoral training grant in Molecular Biophysics and RIM was supported by a grant from the NIH.

References

1. Hendrick, J.P. & Hartl, F.-U. (1995). The role of molecular chaperones in protein folding. *FASEB J.* **9**, 1559–1569.
2. Gething, M.-J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33–45.
3. Morimoto, R.I.,Tissieres, A. & Georgopoulos, C. (1994). *The Biology of Heat Shock Proteins and Molecular Chaperones.* Cold Spring Harbor Laboratory Press, NY.
4. Hatayama, T., Asai, Y., Wakatsuki, T., Kitamura, T. & Imahara, H. (1993). Regulation of hsp70 synthesis induced by cupric sulfate and zinc sulfate in thermotolerant HeLa cells. *J. Biochem.* **114**, 592–597.
5. Yamamoto, N., Smith, M.W., Maki, A., Berezesky, I.K. & Trump, B.F. (1994). Role of cytosolic Ca^{2+} protein-kinases in the induction of the Hsp70 gene. *Kidney Int.* **45**, 1093–1104.
6. Resendez, E., Jr., Ting, J., Kim, S., Wodden, S.K. & Lee, A.S. (1986). Calcium ionophore A23187 as a regulator of gene expression in mammalian cells. *J. Cell. Biol.* **103**, 2145–2152.
7. Tillman, J.B., Mote, P.L., Walford, R.L. & Spindler, S.R. (1995). Structure and regulation of the mouse Grp78 (BiP) promoter by glucose and calcium ionophore. *Gene* **158**, 225–229.
8. Tilly, K., McKittrick, N., Zylicz, M. & Georgopoulos, C. (1983). The DnaK protein modulates the heat-shock response of *Escherichia coli*. *Cell* **34**, 641–646.
9. Zylicz, M., LeBowitz, J.H., McMacken, R. & Georgopoulos, C. (1983). The DnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an *in vitro* DNA replication system. *Proc. Natl. Acad. Sci. USA* **80**, 6431–6435.
10. Flaherty, K.M., Wilbanks, S.M., DeLuca-Flaherty, C. & McKay, D.B. (1994). Structural basis of the 70 kilodalton heat shock cognate protein ATP hydrolytic activity *J. Biol. Chem.* **269**, 12899–12907.
11. Wilbanks, S.M. & McKay, D.B. (1995). How potassium affects the activity of the molecular chaperone Hsc70. *J. Biol. Chem.* **270**, 2251–2257.
12. Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. & Craig, E.A. (1992). The translation machinery and 70 kD heat shock protein cooperate in protein synthesis. *Cell* **71**, 97–105.
13. Beckmann, R.P., Mizzen, L.A. & Welch, W.J. (1990). Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly. *Science* **248**, 850–854.
14. Skowyra, D., Georgopoulos, C. & Zylicz, M. (1990). The *E. coli* dnaK gene product, the Hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* **62**, 939–944.
15. Munro, S. & Pelham, H. (1986). An hsp70-like protein in the ER: identify with the 78 kD glucose-regulated protein and immunoglobulin heavy-chain binding protein. *Cell* **46**, 291–300.
16. Chirico, W.J., Waters, M.G. & Blobel, G. (1988). 70 K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332**, 805–810.
17. Murakami, H., Pain, D., & Blobel, G. (1988). 70 kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* **107**, 2051–2057.
18. Kang, P.-O., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A. & Pfanner, N. (1990). Requirements for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* **348**, 137–143.
19. Shi, Y. & Thomas J.O. (1992). The transport of proteins into the nucleus requires the 70 Kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* **12**, 2186–2192.
20. Wagner, I., Arlt, H., vanDyck, L., Langer, T. & Neupert W. (1994). Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *EMBO J.* **13**, 5135–5145.
21. Stone, D.E. & Craig, E.A. (1990). Self-regulation of 70 kilodalton heat shock proteins in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **10**, 1622–1632.
22. Abavaya, K., Myers, M.P., Murphy, S.P. & Morimoto, R.I. (1992). The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes Dev.* **6**, 1153–1164.
23. Gaut, J.R. & Hendershot, L.M. (1993). The immunoglobulin-binding protein *in vitro* autophosphorylation site maps to a threonine within the ATP binding cleft but is not a detectable site of *in vivo* phosphorylation. *J. Biol. Chem.* **268**, 12691–12696.
24. Leustek, T., Toledo, H., Brot, N. & Weissbach, H. (1991). Calcium-dependent autophosphorylation of the glucose-regulated protein, Grp78. *Arch. Biochem. Biophys.* **289**, 256–261.
25. McCarty, J.S. & Walker, G.C. (1991). DnaK as a thermometer: threonine-199 is site of autophosphorylation and is critical for ATPase activity. *Proc. Nat. Acad. Sci. USA* **88**, 9513–9517.
26. Wu, B., Hunt, C. & Morimoto, R.I. (1985). Structure and expression of the human gene encoding major heat shock protein Hsp70. *Mol. Cell. Biol.* **5**, 330–341.
27. Dworniczak, B. & Mirault, M.E. (1987). Structure and expression of a human gene coding for a 71 kD heat shock 'cognate' protein. *Nucleic Acids Res.* **15**, 5181–5197.
28. Bhattacharyya, T., *et al*., & Morimoto, R.I. (1995). Cloning and subcellular localization of human mitochondrial Hsp70. *J. Biol. Chem.* **270**, 1705–1710.
29. Hunt, C & Morimoto, R.I. (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc. Natl. Acad. Sci. USA* **82**, 6455–6459.
30. Freeman, B.C., Myers, M.P., Schumacher, R. & Morimoto, R.I. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO J.* **14**, 2281–2292.
31. Freeman, B.C. & Morimoto, R.I. (1996). The human cytosolic molecular chaperones Hsp90, Hsp70 (Hsc70), and HDJ-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J.* **15**, 2969–2979.
32. Buchberger, A., *et al.*, & Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J. Biol. Chem.* **270**, 16903–16910.
33. Flaherty, K.M., DeLuca-Flaherty, C. & McKay, D.B. (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346**, 623–628.
34. McKay, D.B., Wilbanks, S.M., Flaherty, K.M., Ha, J.-H., O'Brien, M.C. & Shirvanee, L.L. (1994). Stress-70 proteins and their interaction with nucleotides. In *The Biology of Heat Shock Proteins and Molecular Chaperones.* pp. 152–177, Cold Spring Harbor Laboratory Press, NY.
35. Zhu, X., *et al*., & Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614.
36. Brünger, A.T. (1992). *X-PLOR, Version 3.1. A System for X-ray Crystallography and NMR*. Yale University Press, New Haven, CT.
37. Braig, K., *et al.*, & Sigler, P.B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371**, 578–586.
38. Boisvert, D.C., Wang, J.M., Otwinowski, Z., Horwich, A.L. & Sigler, P.B. (1996). The 2.4 Å crystal structure of the bacterial chaperonin GroEL complexed with ATP-gamma-S. *Nat. Struct. Biol.* **3**, 170–177.
39. Schevitz, R.W., Otwinowski, Z., Joachimiak, A., Lawson, C.L. & Sigler, P.B. (1985). The three-dimensional structure of trp repressor. *Nature* **317**, 782–786.
40. Flaherty, K.M., McKay, D.B., Kabsch, W. & Holmes, K.C. (1991). Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70 kDa heat shock cognate protein. *Proc. Nat. Acad. Sci. USA* **88**, 5041–5045.

41. Buchberger, A., Schroder, H., Buttner, M., Valencia, A. & Bukau B. (1994). A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. *Nat. Struct. Biol.* 1, 95–101.
42. Wawrzynow, A., Banecki, B., Wall, D., Liberek, K. & Georgopoulos, C. (1995). ATP hydrolysis is required for the DnaJ-dependent activation of DnaK chaperone for binding to both native and denatured protein substrates. *J. Biol. Chem.* 270, 19307–19311.
43. Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. & Holmes, K.C. (1990). Atomic structure of the actin:DNAse I complex. *Nature* 347, 37–44.
44. Glusker, J.P. (1990). Structural aspects of metal liganding to functional groups in proteins. *Adv. Protein Chem.* 42, 1–76.
45. O'Brien, M.C., Flaherty, K.M. & McKay, D.B. (1996). Lysine 71 of the chaperone protein Hsc70 is essential for ATP hydrolysis. *J. Biol. Chem.* 271, 15874–15878.
46. O'Brien, M.C. & McKay, D.B. (1993). Threonine 204 of the chaperone protein Hsc70 influences the structure of the active site, but is not essential for ATP hydrolysis. *J. Biol. Chem.* 268, 24323–24329.
47. Rose, T., Sebo, P., Bellalou, J. & Ladant, D. (1995). Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin. *J. Biol. Chem.* 270, 26370–26376.
48. Ferretti, M.E. (1995). Adenylate-cyclase activity from *Hirudo medicinalis* segmental ganglia: modulation by calcium and calmodulin. *Comp. Biochem. Physiol.* 110, 79–86.
49. Otwinowski, Z. & Minor, W. (1995). DENZO, XDISPLAYF, and SCALEPACK: an oscillation data processing suite for macromolecular crystallography. The HJL Manual, Yale University Press, New Haven, CT.
50. Lamzin, V.S. & Wilson, K.S. (1993). Automated refinement of protein models. *Acta Cryst. D* 49, 129–147.
51. Jones, A.T., Zou, J.-Y., Cowan, S.W. & Kjeldgaard, M. (1991). Improved methods for building proteins models in electron-density maps and the location of errors in these models. *Acta Cryst. A* 47, 110–119.