Neutron Crystallographic Analysis of Nucleotide-Binding Domain of Hsp72 in complex with ADP.

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1. Neutron structure of the Hsp72-ADP complex.
2. The 70 kDa heat shock proteins (Hsp70s) are ATP-dependent molecular chaperones that contain the N-terminal nucleotide-binding domain (NBD) and the C-terminal substrate-binding domain. Hsp70s binds to misfolded/unfolded proteins and prevent aggregation of misfolded/unfolded proteins. The ATP hydrolysis reaction in the NBD plays a key role in allosteric control of the bindability to substrate proteins. In the present study, we solved the neutron crystal structure of the NBD of Hsp72, a heat-inducible Hsp70 family member, in complex with ADP to study the structure-function relationship focusing on hydrogens. The H/D exchange analysis revealed the protein flexibility in relation to the conformational change. ADP bound to Hsp72 was fully deprotonated, and the catalytically important residues, including D10, D199 and D206 are also deprotonated. Neutron analysis also enabled the characterization of the water clusters in the NBD. Enzymatic assay and X-ray crystallographic analysis revealed that Y149A mutation exhibited the higher ATPase activity, and caused the disruption of the water cluster and incorporation of an additional magnesium ion. Y149 is suggested to contribute to the low intrinsic ATPase activity and to stabilize the water cluster. Collectively, our structural studies contribute to grasp the molecular basis for the Hsp72 function.
3. Neutron protein crystallography; hsp72; hydrogen-bond network; ATPase; water cluster.
4. Introduction

The 70 kDa heat shock proteins (Hsp70s) are widely conserved molecular chaperone in Eukaryote. Hsp70s are expressed in response to a variety of cellular stress such as heat, UV and oxygen deprivation, and play key roles in regulation of cellular protein homeostasis by protein refolding, protein translocation across organelle and disaggregation of protein aggregates ([Hartl *et al.*, 2011](#_ENREF_12); [Mayer & Bukau, 2005](#_ENREF_21); [Saibil, 2013](#_ENREF_29)). In humans, several paralogs of Hsp70s are found in cytosol, endoplasmic reticulum (ER) and mitochondria. The heat-inducible Hsp70 (Hsp72, a.k.a. HSPA1A and HSPA1B) and the heat shock cognate Hsp70 (Hsc70, a.k.a. HSPA8) are the major cytosolic Hsp70s, while BiP (HSPA5) and mortalin (HSPA9) are localized in ER and mitochondria, respectively ([Vos *et al.*, 2008](#_ENREF_35)). The *Escherichia coli* Hsp70 ortholog DnaK is one of the most well-studied members of the Hsp70 family.

Hsp70 is an ATPase that binds to and releases unfolded and misfolded proteins depending on the nucleotide-binding state. Hsp70 composed of an N-terminal nucleotide-binding domain (NBD), a C-terminal substrate-binding domain (SBD) and a flexible linker that connects NBD and SBD. The NBD can allosterically communicate with the SBD. The binding of ATP to NBD decreases the substrate-binding affinity for the SBD, and the hydrolysis to ADP increases the substrate-binding affinity ([Pierpaoli *et al.*, 1998](#_ENREF_27)). The NMR based structural study of DnaK in complex with ADP and peptide and the crystallographic analysis of DnaK in complex with ATP showed that the ADP-bound state adopts an extended conformation and the binding of ATP in the NBD induces a large structural rearrangements ([Qi *et al.*, 2013](#_ENREF_28); [Bertelsen *et al.*, 2009](#_ENREF_6)). This structural rearrangements composed of the docking of the SBD and the intradomain linker to the NBD and the conformational change in the SBD, resulting in the promotion of substrate protein release ([Zuiderweg *et al.*, 2013](#_ENREF_44)). The substrate binding and release cycle can also be controlled by cochaperones such as Hsp40 and nucleotide exchange factors (NEFs). The J-domain of Hsp40 stimulates the ATPase activity. NMR and molecular dynamics simulation studies indicated that the J-domain predominantly interacts with the NBD in the bacterial Hsp70/Hsp40 system ([Ahmad *et al.*, 2011](#_ENREF_2)), while it still remains to be seen how Hsp40 binds to Hsp70 ([Li *et al.*, 2009](#_ENREF_20); [Alderson *et al.*, 2016](#_ENREF_3)). The eukaryotic NEF Bcl2-associated anthanogene 1 (BAG1) protein directly binds to the NBD of Hsp70 and stimulates the ATPase activity. The binding of BAG1 to Hsc70 induces opening of the nucleotide-binding cleft, promoting dissociation of nucleotides ([Sondermann *et al.*, 2001](#_ENREF_32)).

Hydrolysis of ATP to ADP in the NBD triggers functionally essential cycles of substrate binding and release, and thus the NBD plays a pivotal role during the cycle. X-ray crystal structures of the isolated NBD of human Hsp70 paralogs, including Hsp72, Hsc70, BiP and mortalin, have been determined ([Shida *et al.*, 2010](#_ENREF_31); [Zhang *et al.*, 2015](#_ENREF_43); [Wisniewska *et al.*, 2010](#_ENREF_38); [Amick *et al.*, 2014](#_ENREF_4)). Their overall structures are well conserved with 52-89% sequence identities. The NBD is composed of four subdomains IA, IB, IIA and IIB, and the nucleotide-binding pocket is located at the central part of the subdomains (Figure 1A). Crystallographic analysis showed that ADP simultaneously binds to Hsp70 with catalytic magnesium ions, orthophosphate ions and monovalent cations such as sodium and potassium ions depending on the purification buffer or crystallization additives, while an ATP analogue AMPPnP binds to Hsp70 only with a catalytic magnesium ion ([Shida *et al.*, 2010](#_ENREF_31); [Arakawa *et al.*, 2011](#_ENREF_5)). While the monovalent ions are required for optimal ATPase activity ([Wilbanks & McKay, 1995](#_ENREF_37); [O'Brien & McKay, 1995](#_ENREF_26)), and the presence orthophosphate ion increases the binding affinity of Hsp70 for ADP ([Arakawa *et al.*, 2011](#_ENREF_5)). The ATPase reaction is a hydrolysis using a water molecule as a nucleophile. The enzymatic catalysis therefore can be affected by not only the binding affinity for ATP but also various circumstances such as the static stability, water accessibility and long-range electrostatic interaction. So far, X-ray crystallographic, biophysical and mutagenic analyses of the NBD of mutated Hsp70 were widely adopted to investigate the molecular features that are involved in the intrinsic ATPase activity ([Arakawa *et al.*, 2011](#_ENREF_5); [O'Brien *et al.*, 1996](#_ENREF_24); [O'Brien & McKay, 1993](#_ENREF_25); [Ha *et al.*, 1997](#_ENREF_11); [Moseng *et al.*, 2019](#_ENREF_22)). In addition to these, we thought neutron protein crystallography would be a good approach to understand the molecular details of the ATPase from different points of view and perspectives.

Neutron protein crystallography is useful technique to determine the position of hydrogen atoms ([Niimura & Bau, 2008](#_ENREF_23)). Neutrons are scattered from nuclei, and neutron scattering lengths for hydrogen and deuterium are comparable with those for carbon, nitrogen and oxygen. Hydrogen/deuterium exchanged or perdeuterated crystals are generally used for neutron protein crystallography, because hydrogen atoms scatters with a strong incoherent scattering component, resulting in high backgrounds ([Howard *et al.*, 2016](#_ENREF_14); [Dajnowicz *et al.*, 2017](#_ENREF_7); [Schiebel *et al.*, 2018](#_ENREF_30); [Yokoyama *et al.*, 2019](#_ENREF_40)). Neutron crystallography, therefore, can determine protonation states as deuteriums, direction of heavy waters (D2O) and hydrogen/deuterium exchange ratio even at moderate resolution, although the neutron diffraction experiments require large crystals in comparison to the X-ray diffraction experiments.

In the present study, we describe the neutron crystal structure of the NBD of human major inducible Hsp72 in complex with ADP at 2.2 Å resolution. We will fully characterize the protonation states in the ADP-binding pocket, protein flexibility by H/D exchange ratios and two water clusters in the NBD. In addition, the neutron structure motivated us to investigate the role of Y149 residue that is associated with the hydrogen bond network and the water cluster. Together with the results of the neutron crystallographic analysis, we will discuss the ATPase activity of Hsp72 in relation to the hydrogen-bond networks and water clusters in the protein.

1. Materials and methods
   1. X-N joint refinement

We have previously collected the room temperature neutron and X-ray diffraction data of the nucleotide-binding domain of HspA1B (Hsp72) in complex with ADP at 2.2 Å and 1.6 Å resolution ([Yokoyama *et al.*, 2017](#_ENREF_41)). Prior to the X-N joint refinement, the structure was refined using X-ray data. The structure was solved by molecular replacement using as a search model of the Hsp72 complexed with an inhibitor (PDB 5AQZ) using MOLREP ([Vagin & Teplyakov, 1997](#_ENREF_33)). After the several cycles of X-ray structure refinement with manual correction by COOT, the X-N joint refinement was initiated from the refined structure using PHENIX.REFINE ([Emsley & Cowtan, 2004](#_ENREF_8); [Adams *et al.*, 2010](#_ENREF_1)). The hydrogen and deuterium atoms were stepwisely included in order of exchangeable sites, unexchangeable sites and heavy water molecules. The final *R*cryst and *R*free factors for the neutron data were 18.4% and 22.2%, respectively. The final *R*cryst and *R*free factors for the X-ray data were 16.7% and 19%, respectively. The coordinates and structure factors have been deposited at the Protein Data Bank under the PDB code XXX1. Crystal and refinement data were summarized in Table 1.

* 1. Protein production

The expression plasmid of the NBD of Y149A mutated Hsp72 was prepared with the plasmid pET-22b(+) of the NBD of WT-HspA1B (residues 1−380) as a template using the QuikChange Site-Directed Mutagenesis Kit. The expression and purification of WT-Hsp72 and Y149A-Hsp72 was carried out as previously reported ([Yokoyama *et al.*, 2017](#_ENREF_41)). In brief, the recombinant plasmid was transformed into BL21(DE3). Protein expression was induced with 0.25 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The cells incubated at 293 K overnight with agitation at 180 rev min−1. The harvested cells were resuspended, lysed by sonicated, and the cell lysate was centrifuged at 9400*g* for 60 min at 277 K. A Metal-affinity chromatography using Ni-NTA was performed, and the desired protein fraction was pooled. The protein sample was dialysed against a buffer [20 mM Tris-HCl pH 8.0 and 100 mM NaCl]. The protein sample was then applied to an affinity chromatography using Blue Sepharose 6 Fast Flow resins. The protein was eluted with a buffer [20 mM Tris-HCl pH 8.0 and 1 M NaCl], and the elution fraction was dialysed against a buffer [20 mM Tris-HCl pH 8.0 and 100 mM NaCl].

The recombinant plasmids pET-22b(+)-full-length Hsp72 (residues 1−641), DNAJ homolog subfamily B member 1 (DnaJB1, residues 1−340) and the BAG domain of BAG family molecular chaperon regulator 1 (BAG1, residues 151-261) were synthesized by GenScript. The plasmids were transformed into SoluBL21 and the transformed cells were grown at 310K to an OD600 of 0.4−0.6, and then the expression of the C-terminally 6His-tagged proteins were induced overnight at 293 K with 0.25 mM IPTG. The cell pellets were lysed using sonication, and the supernatant was purified using Ni-affinity chromatography followed by size exclusion chromatography using a Superdex 200 gel-filtration column equilibrated with a buffer [20 mM Tris-HCl pH 8.0 and 100 mM NaCl]. Protein aliquots were concentrated to 10-20 mg/mL using an Amicon Ultra-10 centrifugal device (10 kDa cutoff), flash-frozen using liquid nitrogen, and stored at 243 K. The expression plasmids of full-length Y149A-mutated Hsp72 and full-length T204A mutated Hsp72 were prepared with the plasmid of full-length WT-Hsp72 as a template using the QuikChange Site-Directed Mutagenesis Kit. Identical expression and purification schemes were performed for full-length Y149A-mutated Hsp72 and full-length T204A mutated Hsp72.

* 1. ATPase activity measurement

ATPase enzymatic activities of full-length Hsp72 were assessed by a colorimetric malachite green assay in which orthophosphates ion can be detected as previously described ([Yokoyama *et al.*, 2021](#_ENREF_42)). The ATPase reaction was directly performed in microplate wells using 40 μL of sample composed of 0.3 μM full-length Hsp72 (WT, Y149A or T204A), 40 mM Tris-HCl pH 8, 50 mM KCl, 10 mM MgCl2, and 15-1000 μM ATP in the presence or absence of co-chaperons DnaJB1 and BAG1. The reaction samples were incubated at 310 K for 180 min (WT and T204A) or 40 min (Y149A), and then stained by addition of 160 μL of malachite green reagent composed of one volume of 4.2% (*w*/*v*) ammonium molybdate in 4 *N* HCl and three volumes of 0.045% (*w*/*v*) malachite green. The absorbance at 620 nm was measured using a FilterMax F5 (Molecular Devices). The values of Michaelis constant *K*m and *K*cat were determined by the Lineweaver-Burk double reciprocal plot for ATP as a substrate.

* 1. X-ray protein crystallography

Single crystals of the NBD of Y149A-Hsp72 in complex with ADP were obtained by equilibrating a droplet composed of 12.6 mg/mL Y149A-Hsp72-NBD, 2 mM ADP, 15.5% Polyethylene Glycol Monomethyl Ether 550 (PEG550 MME), 100 mM MgCl2, 150 mM KCl and 50 mM Bis-Tris pH 6.0 against a crystallization buffer composed of 31% PEG550 MME, 200 mM MgCl2, 300 mM KCl and 100 mM Bis-Tris pH 6.0. Although Y149A-Hsp72-NBD in complex with AMPPnP was also crystallized under the crystallization condition for Y149A-Hsp72-ADP complex, single crystals appropriate for X-ray diffraction experiment were not obtained. We therefore adopted a micro-seeding method to produce single crystals. Parent crystals were obtained by equilibrating a droplet composed of 12.9 mg/mL Y149A-Hsp72-NBD, 2 mM AMPPnP, 14.5% Polyethylene Glycol Monomethyl Ether 550 (PEG550 MME), 100 mM MgCl2 and 50 mM Tris-HCl pH 7.0 against a crystallization buffer composed of 29% PEG550 MME, 200 mM MgCl2 and 100 mM Tris-HCl pH 7.0. The crystals were directly crushed in the droplet using a needle, and the seed stock was prepared by 1000-fold dilution of the droplet with additional reservoir solution. Finally, single crystals of Y149A-Hsp72 in complex with AMPPnP were obtained under the crystallization condition [29% PEG550 MME, 200 mM MgCl2 and 100 mM Tris-HCl pH 7.0] using the droplet composed of 1.9 μL of 16.1 mg/mL Y149A-Hsp72, 1.9 μL of the reservoir solution and 0.2 μL of the seed stock. Crystals were begun to appear within a few hours, stopped growing after 1 week. The crystals were cryo-protected by 33% PEG550 MME, and directly stored in liquid nitrogen until X-ray diffraction experiment.

Cryogenic X-ray diffraction data were collected at beamline BL-17A at the Photon Factory in Japan, and processed with *XDS* ([Kabsch, 2010](#_ENREF_16)). The Y149A-Hsp72 crystals belonged to space group *P*212121 and were isomorphous with the room temperature WT-Hsp72 crystals. Therefore, structure refinement could be carried out using the coordinates of the room temperature WT-Hsp72 without performing Patterson-based molecular replacement. Several rounds of iterative model building and refinement were performed using *Coot* and *PHENIX.REFINE* ([Emsley & Cowtan, 2004](#_ENREF_8); [Adams *et al.*, 2010](#_ENREF_1)). The coordinates and structure factors of Y149A-Hsp72-ADP and Y149A-Hsp72-AMPPnP have been deposited at the Protein Data Bank under the PDB code XXX2 and XXX3, respectively. Crystal and refinement data were summarized in Table 2.

1. Results
   1. Overall neutron structure of WT-Hsp72 in complex with ADP

In the previous study, the neutron diffraction data and X-ray diffraction data of the NBD of Hsp72 (residues 1-380) in complex with ADP were collected at room temperature using the BIODIFF diffractometer at Forschungs-Neutronenquelle Heinz Maier-Leibnitz (FRM II, Germany) and the beamline AR-NW12A at the Photon Factory (Japan), respectively ([Yokoyama *et al.*, 2017](#_ENREF_41)). The neutron structure was jointly refined with the X-ray data at 2.2 Å resolution, resulting in the *R*cryst of 18.4 % and an *R*free of 22.2% (Table 1). The asymmetric unit contains one molecule of Hsp72 and ADP, 86 heavy water molecules, one magnesium ion and one sodium ion (Figure 1a). Our neutron structure was superposed on the previous structure at cryo temperature (PDB 3ATU) with an RMSD of 0.28 Å, indicating that the structures at room temperature and cryo temperature were comparable. The ADP molecule was clearly observed in the neutron scattering length map and electron density map, although the neutron scattering length density was partially cancelled by neighboring H atoms (Figure 1b). While the neutron scattering density map omitting H and D atoms clearly showed the presence of the H and D atoms of adenosine moiety, any peak was not observed around the phosphate groups, indicating that the phosphate group of ADP was fully deprotonated. The NBD of Hsp72 isoforms in complex with ADP are often crystallized with an inorganic phosphate ion derived from the expression host ([Wisniewska *et al.*, 2010](#_ENREF_38)). However, the inorganic phosphate was not observed in our neutron structure. The inorganic phosphate ions may be released during the crystallization, because it was required for 10 weeks to obtain large crystals for neutron diffraction experiment ([Yokoyama *et al.*, 2017](#_ENREF_41)). We found three water clusters in the Hsp72-ADP complex (cluster 1-3) (Figure 1a). The water cluster 1 was associated with the binding of the phosphate group of ADP. The water cluster 2 was located between the subdomain IA and IB apart from the ADP binding site. The water cluster 3 was located at the interface of the subdomain IA and IIA near the cluster 1, but the water cluster 1 and 3 were not connected by hydrogen bonds.

The H/D exchange ratio could be determined because the H/D exchanged crystals were used. In particular, the H/D exchange ratio of amide protein is generally useful to assess the protein dynamics, rigidity and water accessibility. While the H/D unchanged residues (H>80%) were primarily observed in the subdomains IA and IIA, the H/D exchanged residues (D>80%) were thoroughly observed in whole protein (Figure 1c, Table 2). The H/D unchanged residues were located in the hydrophobic core in the subdomains IA and IIA. The average *B*-factors of the subdomains IA and IIA were lower than those of subdomains IB and IIB, suggesting that the formation of hydrophobic core by the unexchanged residues is likely to contribute the statical stability of the subdomains.

* 1. ADP binding

ADP binds to the NBD with the high affinity and the dissociation rate is known to be slow ([Hohfeld & Jentsch, 1997](#_ENREF_13); [Arakawa *et al.*, 2011](#_ENREF_5)). A detail illustration of the interactions between Hsp72 and ADP may provide the better understanding of the ADP-binding event. The adenosine moiety located at the interface of subdomains IIA and IIB. 1-N and 3-N accepted hydrogen bonds from S275 and a water molecule, respectively, and the water molecule also donated a hydrogen bond to S340, bridging the hydrogen-bond network between the adenyl group and S340 (Figure 2a, supplementary Figure S1a). 6-NH2 was located at the surface, and any specific interaction was not observed between 6-NH2 and Hsp72. Although the adenyl ring was located in the middle of the guanidyl groups of R272 and R342, they were too far to form a π‧‧‧π stacking. The ribose was stabilized by hydrogen bonds of 2’-OH and 3’-OH. E268 adopted a dual conformation. The one conformation was involved in the salt bridge with the side chain of R272 and the other conformation was involved in the hydrogen bond with 2’-OH. 2’-OH also accepted a hydrogen bond from K271 that forms a salt bridge with D264. 3’-OH donated a hydrogen bond to a water molecule that accepted a hydrogen bond from D264. In summary, the adenyl and ribose groups were involved in three and five hydrogen bonds, respectively.

The diphosphate moiety bound at the interface of the subdomains IA, IIA and IIB with highly hydrated state by the water cluster 1 (Figure 1a). As previously reported, one magnesium ion and one sodium ion were observed. The magnesium ion is octahedrally coordinated by the oxygen atoms from w1-w5 and β-phosphate group (Figure 2b). The sodium ion is tetrahedrally coordinated by the oxygen atoms from w6, sidechain of D10, mainchain of Y15 and β-phosphate group. Because the peaks of the deuterium atoms of the water were clearly observed in the neutron scattering density map, all water molecules coordinated in the form of D2O but not DO− (Supplementary Figure S1b). There are three aspartate residues (D10, D199 and D206) and one glutamate residue (E175) in the pocket, and they were in the deprotonated states. D199 was accepted a hydrogen bond from w1. w1 was also donated a hydrogen bond to E175 that formed a salt bridge with K71. These residues are considered to be catalytic residues for the ATPase activity. T204 was accepted a hydrogen bond from w7 and donated a hydrogen bond to w7, participating with the large hydrogen bond network composed of w4, w7, w8 w79, K71 and β-phosphate group. This hydrogen bond network expanded to Y149 via w29 and D206. The binding of diphosphate group to Hsp72 was involved in more than twenty of hydrogen bonds and salt bridges.

* 1. Water cluster

The water cluster 2 was composed of seven water molecules (w12-18) and they were statically quite stable with an average B-factor of 21.4 Å2 (Figure 3a, Supplementary Figure S2a). The water cluster 2 was mostly surrounded by the hydrophobic residues such as L11, A70, I74, I119, V123, I144, V146, F150 and the hydrophobic moiety of K71, and thus the role of the water cluster is appeared to cancel the polarity inside the cavity through hydrogen-bonding. While w15, w17 and w18 hydrogen-bonded in a tetrahedral coordination (two hydrogen bonds as donors and two hydrogen bonds as acceptors), w12, w13, w14 and w16 hydrogen-bonded in a triangle coordination (two hydrogen bonds as donors and one hydrogen bond as acceptors). The polarities of carbonyl oxygen of L11, G12, T13, S40 and T145 were neutralized by hydrogen bonds that were donated from w12, w15, w17, w18 and w12, respectively. In addition, w13 was donated a hydrogen bond to T158. The neutron scattering density map indicated that the sidechain of S120 adopted a dual conformation, donating hydrogen bonds to W14 and W16 (Supplementary Figure S2a). The average hydrogen bond length between the water molecules in the cluster were 1.93 Å, and the average hydrogen bond length between the water the water molecules and the amino acid residues was 2.09 Å. These results imply that the water molecules were tightly packed in the pocket, but the protein held the water cluster by weaker hydrogen bonds.

The water cluster 3 was composed of seven water molecules (w19-24) and they were statically stable with an average *B*-factor of 27.0 Å2 (Figure 3b). This water cluster was contacted with the polar residues (E175, D199, D206 and S208) and the hydrophobic residues (P147, A148, P176 and I197). Although the three acidic residues were all deprotonated, the boomerang-shaped neutron maps were observed for the water molecules, indicating that they were bound as D2O form but not as hydronium ions (Supplementary Figure S2b). Only w23 hydrogen-bonded in a tetrahedral coordination and the other water molecules hydrogen-bonded in a triangle coordination. The lack of the tetrahedral coordination may contribute to the relative instability in comparison to the water cluster 2. The D‧‧‧O distance between w24 and w23 was 2.2 Å and slightly longer than those between the other water molecules (1.9 Å). The E175 and D206 were accepted hydrogen bonds from w23, and D199 was accepted a hydrogen bond from w24. This water cluster was stabilized by more than 15 hydrogen bonds and expanded to w19 that was located at the entrance of the pocket. E175 is considered to be a catalytic residue that supplies a nucleophilic water by the deprotonation. The role of the water cluster is likely to stabilize the structure and to supply a nucleophilic water but not to cancel the extra negative charge of the aspartate and glutamate residues.

It is frequently observed that solvent position and protein conformation are different between the cryogenic and room temperature structures ([Kwon *et al.*, 2018](#_ENREF_19)). Our neutron structure of Hsp72-ADP was compared with the cryogenic X-ray structures of the Hsp72-AMPPnP (PDB 2E8A) and Hsp72-ADP complexes (PDB 3ATU) ([Shida *et al.*, 2010](#_ENREF_31); [Arakawa *et al.*, 2011](#_ENREF_5)). The neutron Hsp72-ADP structure was superimposed on the cryo Hsp72-AMPPnP and Hsp72-ADP structures with RMSD values of 0.39 and 0.28 Å, respectively, indicating that the overall structures were almost identical (Supplementary Table S1, Supplementary Figure S3a, b). In addition, the water clusters 2 and 3 were conserved in the cryogenic Hsp72-AMPPnP and Hsp72-ADP structures. In the ADP complex, an inorganic phosphate ion was bound to the ATP-binding pocket and hydrogen-bonded to T204 and Y149 via a water molecule, but the presence of phosphate ion had no effect on the position of the water molecules of the water cluster 3 (Supplementary Figure S3b). These comparisons indicate that the nucleotides and temperature had no effect on the water clusters 2 and 3.

* 1. ATPase activity of Y149A and T204A mutant

Y149 is located at the interface of the subdomain IA and IIA, and involved in the conformational change between ATP- and ADP-bound state. Y149 was participated in the hydrogen bond network of the water cluster 1, and also contacted with the water cluster 3. Y145A mutation in DnaK (corresponding to Y149 in Hsp72) increased the ATP hydrolysis rate ([Kityk *et al.*, 2015](#_ENREF_18)). In addition to our neutron crystallographic analysis, this background prompted us to investigate the role of Y149 by determination of the ATPase kinetic parameters and X-ray crystal structure of Y149A mutated Hsp72. We also determined the kinetic parameters of T204A mutated Hsp72 (T204A-Hsp72). T204A mutation has been shown to be an ATPase deficient mutation in DnaK and BiP, and therefore we considered that the experiments using T204A-Hsp72 would be suitable for negative control experiments ([Yang *et al.*, 2015](#_ENREF_39); [Qi *et al.*, 2013](#_ENREF_28)).

We adopted the Lineweaver-Burk plot to determine the kinetic parameters (*K*m and *K*cat) for the full length Hsp72 using malachite green assay in which orthophosphate ions can be directly detected. The Y149A mutation caused an increase of *K*cat and had no effect on *K*m (Figure 4, Table 3). The T204A mutation decreased *K*cat and increased *K*m. In addition of the determination of the basal activity, we also determined the kinetic parameters in the presence of cochaperones Hsp40 (DnaJB1) or BAG1. The *K*cat value of WT-Hsp72 was increased in a DnaJB1-dose-dependent manner. The *K*cat and *K*m values were decreased in a BAG1-dose-dependent manner, and the ATPase activity (*K*cat/*K*m) was not significantly changed. The *K*cat value of Y149A-Hsp72 was also increased by the addition of DnaJB1, but the stimulation of the ATPase activity was much stronger than WT-Hsp72. On the other hand, the ATPase activity of T204A-Hsp72 was not stimulated by DnaJB1. The presence of BAG1 drastically decreased the *K*m value and slightly increased the *K*cat value, recovering the ATPase activity comparable with WT-Hsp72. In summary, Y149A-Hsp72 and T204A-Hsp72 are more sensitive to DnaJB1 and BAG1, respectively.

* 1. X-ray crystal structures of Y149A-Hsp72 in complex with ADP or AMPPnP

X-ray crystal structures of the NBD of Y149A-Hsp72 in complex with ADP and AMPPnP at cryogenic temperature were solved at 1.8 Å and 1.7 Å resolution, respectively (Table 4). Y149A-Hsp72 crystals were isomorphous to WT-Hsp72 crystals with the space group of *P*212121. The overall structures of the cryogenic Y149A-Hsp72-ADP/AMPPnP structures were ideally identical to the room temperature WT-Hsp72-ADP and the cryogenic WT-Hsp72-ADP/AMPPnP with the RMSD values of 0.21-0.38 Å, indicating that the mutation had no effect on the overall structure (Supplementary Table S1).

As with the WT-Hsp72-ADP structure, an inorganic phosphate ion was observed in the Y149A-Hsp72-ADP structure (Figure 5a, Supplementary Figure S4a). This ion was hydrogen-bonded to E175 and T204 and coordinated to the magnesium ion. While the water molecules in cluster 3 was conserved in Y149A-Hsp72-ADP except for w19 which is located at the molecular surface, the network of hydrogen bonds were reorganized due to the movement of w23. w23 was hydrogen-bonded to the phosphate ion and D206. The Y149A mutation introduced three water molecules (w26-w28), forming an alternative hydrogen bond network composed of w20-w22, w26-w28, D206 and the phosphate ion.

Two magnesium ions (Mg1 and Mg2) were found in the Y149A-Hsp72-AMPPnP structure. Mg1 was hexahedrally coordinated by the β-phosphate of AMPPnP, D10 and two water molecules (Figure 5b). The position of Mg1 in Y149A-Hsp72-AMPPnP was slightly different from that in WT-Hsp72-AMPPnP. Mg1 in WT-Hsp72-AMPPnP was tetrahedrally coordinated by the β-phosphate of AMPPnP, D10, carbonyl group of Y15 and a water molecule (Supplementary Figure S4b). The additional magnesium ion (Mg2) was found only in the Y149A-Hsp72-AMPPnP structure. Mg2 was octahedrally coordinated by T204, D206 and the four water molecules. While w20-22 and w24 and w25 were conserved in the Y149A-AMPPnP structure, w23 was kicked out by Mg2, disrupting the water cluster 3. The mutation of Y149A induced the formation of the hydrogen bond network composed of w20-w22, w26, w27, w29, w30, Q154 and D206. These hydrogen bonds would stabilize the binding of Mg2. The water molecules w31 and w32, that were coordinated to the magnesium ion, was hydrogen-bonded to E175 and γ-phosphate of AMPPnP. The additional magnesium ion was not observed in the cryogenic WT-Hsp72-AMPPnP structure. The proximity of the magnesium ion to E175 and γ-phosphate group would be responsible for the increased ATPase activity of Y149A-Hsp72.

1. Discussion

The ATPase activity of Hsp70s is basally low and highly controlled by cochaperones, such as Hsp40s and NEFs, and the SBD. As the molecular mechanisms of the intrinsically low ATPase activity of Hsp70s remains still unclear, it is often hard to predict accurately the degree of the enzymatic activity from the structure and sequences. Because there are a variety of microenvironmental factors that modulate the enzymatic activity, illustration of catalytic reaction requires multifaceted biochemical and biophysical studies. In the present study, we determined the neutron crystal structure of the NBD fragment of Hsp72 in complex with ADP. Observation and location of hydrogens and deuteriums enabled a description of the more “complete” structure and provided unique insights into the protein properties related to the ATPase activity.

Hydrogen/deuterium exchange ratio analysis revealed that the subdomains IA and IIA were significantly more rigid than the subdomains IB and IIB (Figure 1c, Table 2). These feature is very likely to reflect a possible conformational change. The NBD in complex with ADP adopts the close conformation and the binding of BAG proteins to the subdomains IB and IIB of the NBD induces the transformation to the open conformation ([Shida *et al.*, 2010](#_ENREF_31); [Sondermann *et al.*, 2001](#_ENREF_32)). The crystallographic analysis of the Hsc70-BAG1 complex revealed that the binding of the BAG protein to Hsc70 resulted in the movement of the subdomain IIB away from the subdomain IB. The subdomain IB and IIB may be intrinsically flexible to accept the binding of BAG proteins.

As a result of careful inspection, we found that ADP and the aspartate residues in the ADP-binding pocket (D10, D199 and D206) were fully deprotonated (Figure 2b). The deprotonation of the aspartate residues appears to be relevant to the charge balance. There are four positive charges of Na+, MG2+ and -NH3+ of K71 in the ADP-binding pocket. The metal ions are required to increase the electron-withdrawing property of the phosphate group and stabilize the binding of the nucleotide. K71 forms a salt bridge with the catalytic residue E175 and maintains the deprotonation state of E175 that is considered to be responsible to deprotonate a nucleophilic water ([O'Brien *et al.*, 1996](#_ENREF_24); [Johnson & McKay, 1999](#_ENREF_15); [Wilbanks *et al.*, 1994](#_ENREF_36); [Flaherty *et al.*, 1994](#_ENREF_10)). While it remains to be seen the protonation states in the ATP (or ATP analogue) complex structure, the deprotonation state of ADP and the acidic residues would stabilize the binding of the metal ions and protonated state of K71.

We characterized the water clusters 2 and 3 in the neutron structure (Figure 3a, 3b). The water cluster 2 was located in the highly hydrophobic pocket that is separated from the nucleotide-binding pocket. In the H/D exchanged crystals, D2O molecules in hydrophobic environment may be blurred due to the cancellation effects of the negative scattering signal of hydrogen atoms ([Fisher *et al.*, 2014](#_ENREF_9)). The water molecules of the cluster 2 were clearly observed as boomerang shape with the quite low *B*-factors, suggesting that these water molecules were exceptionally stable at the hydrophobic pocket. The formation of the water cluster 3 seems to be more relevant to the Hsp70 function, because the catalytically important residues such as E175, D199 and D206 are involved in the formation of the cluster ([Flaherty *et al.*, 1994](#_ENREF_10)). In addition, P147 and Y149 hydrophobically enclosed the water cluster. This proline residue in DnaK (P143) has been suggested to play a key role in allosteric regulation by stabilizing the conformation of the SBD ([Vogel *et al.*, 2006](#_ENREF_34)), and Y145 in DnaK (corresponding to Y149 in Hsp72) also contribute to the allosteric signal by stabilizing the conformation of P143 ([Kityk *et al.*, 2015](#_ENREF_18)). Our present results clearly showed that Y149A mutation increased the intrinsic ATPase activity and the sensitivity for Hsp40 (DnaJB1), indicating that Y149 is also involved in the catalytic reaction and the stimulation by Hsp40. This tyrosine is also located on the pathway from the binding interface of DnaK and DnaJ (bacterial Hsp40) to the ATPase catalytic center ([Kityk *et al.*, 2018](#_ENREF_17)). X-ray crystallographic analysis revealed that the Y149A mutation disrupted the water cluster 3 and caused the binding of the additional cation (Mg2) in the AMPPnP complex. Although there is no direct evidence, the physicochemical property of the water cluster 3 may affect the ATPase activity and the stabilization of the allosteric conformation of SBD.

Crystallographic analysis of Y149A-Hsp72 in complex with AMPPnP elucidated the binding of two magnesium ions (Mg1 and Mg2). Mg1, that is commonly observed in Hsp70 structures, would stabilize the negative charge on the β-phosphate of ATP by the strong electron-withdrawing property. On the other hand, distal Mg2 would stabilize E175, T204. E175 is considered to be the general base that deprotonates the nucleophilic water. The proximity of E175 and Mg2 would stabilize the deprotonated state of E175. The coordination of Mg2 to T204 would also stabilize the hydrogen bond between T204 and the γ-phosphate. The presence of Mg2 indirectly make the γ-phosphate more polarized and susceptible to nucleophilic attack by the hydrolyzing water molecule. Taken together, Y149 hydrophobically holds the water cluster 3 and suppresses the incorporation of additional cations, contributing to the intrinsic low ATPase activity. Currently, crystallization of WT-Hsp72 in complex with AMP-PnP for neutron diffraction experiments is underway. The neutron crystallography of the ATP derivative complex will provide the detailed molecular aspect the enzyme catalysis.

1. Conclusions

ATPase is widely distributed throughout organisms, and one of the most fundamental enzyme. Our study presents the first neutron crystal structure of Hsp72 in complex with ADP. This analysis provides information on the protein flexibility that proves the previously reported conformational change. The acidic residues in the nucleotide-binding pocket and phosphate groups of ADP are observed as fully deprotonated states. It is proposed that the water cluster 3, that is located near the nucleotide-binding pocket, is associated with the ATPase activity and structural stability. ATPase activity measurement and X-ray crystallographic analysis of Y149A-Hsp72 indicate that Y149 contribute to keep the low intrinsic ATPase activity and stabilize the water cluster 3. The present study underlined the significance and usefulness of neutron protein crystallography for the investigation of enzyme-catalyzed reactions.



1. (a) Ribbon plot of the overall neutron structure of the NBD of Hsp72 in complex with ADP (PDB code XXX1). The subdomain IA, IB, IIA and IIB are colored cyan, split pea, green and light blue, respectively. The ADP molecule and the side chain of T204 and Y149 are shown in stick model. The water cluster 1, 2 and 3 are shown in stick and semitransparent surface model, and colored grey, magenta and orange, respectively. (b) Top: The |*F*o|-|*F*c| neutron scattering density map omitting ADP contoured at +4σ. Bottom: The |*F*o|-|*F*c| electron density map omitting ADP contoured at +4σ (blue) and |*F*o|-|*F*c| neutron scattering density map omitting H and D atoms contoured at +3.3σ (green) and -3.3σ (red). (c) The H/D exchanged and unexchanged regions were projected in the ribbon model. The H/D exchanged and unexchanged regions were colored blue and red, respectively.



1. Atomic interactions between Hsp72 and ADP in a wall-eyed stereorepresentation for the adenyl group and ribose moiety (a), and the phosphate moiety (b) (PDB code XXX1). The carbon atoms of ADP and the subunit IA, IIA and IIB are colored dark grey, cyan, green and light blue, respectively. The hydrogen (deuterium), nitrogen, oxygen, sodium, magnesium and phosphorous atoms are coloured while, blue, red, purple, yellow green and orange, respectively. The black dashed lines indicate hydrogen bonds.



1. Wall-eyed illustration of the hydrogen-networks of the water cluster 2 (w12-w18) (a) and water cluster 3 (w19-w26) (b) (PDB code XXX1). The color scheme is the same as in Figure 1 and 2.



1. The line-weaver-Burk plot for the ATPase activity of WT-Hsp72 in the presence of DnaJB1 (a), Y149A-Hsp72 in the presence of DnaJB1 (b) and T204A-Hsp72 in the presence of BAG1. Standard error bars and regression lines are drawn based on the mean of five independent experiments. cluster 3 (b) (PDB code XXX1).



1. X-ray crystal structure of Y149A-Hsp72 in complex with ADP (PDB code XXX2) (a) and Y149A-Hsp72 in complex with AMPPnP (PDB code XXX3) (b). The color scheme is the same as in Figure 1 and 2. The side chain of Y149 and water cluster 3 of the superposed neutron structure (PDB code XXX1) are shown in semitransparent stick model.
2. Statistics on the X-N joint refinement of the NBD of Hsp72.

|  |  |  |
| --- | --- | --- |
|  | Neutron | X-ray |
|  | BIODIFF, FRM II | AR-NW12A, PF |
| Temperature | Room temperature | Room temperature |
| Space group | *P*212121 | *P*212121 |
| *a*, *b*, *c* (Å) | 46.7, 64.6, 145.7 | 46.7, 64.6, 145.6 |
| Resolution range (Å) | 39.3-2.20 (2.26-2.20) | 44.45-1.60 (1.66-1.60) |
| No. of unique reflections | 20952 (1673) | 57639 (5579) |
| Completeness (%) | 90.4 (88.5) | 97.5 (95.4) |
| Redundancy | 2.3 (2.1) | 5.7 (5.9) |
| 〈 *I*/σ(*I*)〉 | 8.1 (2.9) | 13.5 (2.4) |
| *R*meas | 0.117 (0.374) | 0.081 (0.733) |
| Wilson plot *B* factor (Å2) | 23.3 | 22.6 |
| Final *R*cryst | 18.4 | 16.7 |
| Final *R*free | 22.2 | 19.0 |
| No. of non-H atoms | 3056 | |
| No. of H/D atoms | 2956/841 | |
| No. of Water (D2O, DO, O) | 74, 9, 3 | |
| R.m.s. deviations |  |  |
| Bonds (Å) | 0.007 | |
| Angles (°) | 0.99 | |
| Average *B* factors (Å2) |  |  |
| Protein | 31.1 | |
| ADP | 20.2 | |
| Ion | 19.0 | |
| Water | 28.4 | |
| Ramachandran plot |  |  |
| Most favoured (%) | 99.5 | |
| Allowed (%) | 0.5 | |
| PDB ID | XXX1 | |

1. The number of H/D exchanged and unexchanged residues.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Subdomain | No. of residue | H>80% | D>80% | Average *B*-factor (Å2) |
| Overall | 366 | 64 (17.5) | 178 (48.6) | 31.3 |
| IA | 124 | 38 (30.6) | 50 (40.3) | 28.4 |
| IB | 72 | 2 (2.8) | 37 (51.3) | 32.3 |
| IIA | 92 | 20 (21.7) | 48 (52.2) | 28.8 |
| IIB | 78 | 4 (5.1) | 43 (55.1) | 37.3 |

The numbers in parentheses are rate.

1. Kinetic parameters for the ATPase activity of WT-, Y149A- and T204A-Hsp72 in the presence and absence of cochaperones.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Hsp72 | Cochaperone | *K*cat/*K*m (µM−1 h−1) | *K*m (µM) | *K*cat (h−1) |
| WT | None | 1.4 ± 0.0027 | 16 ± 1.8 | 22 ± 0.03 |
| WT | 0.1 µM DnaJB1 | 1.3 ± 0.072 | 37 ± 5.4 | 44 ± 3.8 |
| WT | 0.3 µM DnaJB1 | 1.5 ± 0.072 | 36 ± 4.7 | 53 ± 4.8 |
| WT | 1.0 µM DnaJB1 | 1.9 ± 0.12 | 35 ± 5.3 | 64 ± 6.9 |
| WT | 0.1 µM BAG1 | 1.7 ± 0.28 | 17 ± 2.7 | 27 ± 2.2 |
| WT | 0.3 µM BAG1 | 2.2 ± 0.39 | 13 ± 1.8 | 25 ± 1.6 |
| WT | 1.0 µM BAG1 | 1.9 ± 0.26 | 11 ± 0.78 | 19 ± 1.7 |
| Y149A | None | 2.1 ± 0.43 | 19 ± 3.1 | 35 ± 3.0 |
| Y149A | 0.1 µM DnaJB1 | 5.8 ± 0.78 | 14 ± 2.4 | 79 ± 5.5 |
| Y149A | 0.3 µM DnaJB1 | 6.8 ± 0.99 | 16 ± 2.7 | 100 ± 4.8 |
| Y149A | 1.0 µM DnaJB1 | 8.9 ± 0.32 | 15 ± 2.2 | 140 ± 16 |
| Y149A | 0.1 µM BAG1 | 2.3 ± 0.50 | 18 ± 4.2 | 37 ± 1.7 |
| Y149A | 0.3 µM BAG1 | 1.3 ± 0.12 | 14 ± 1.4 | 19 ± 1.2 |
| Y149A | 1.0 µM BAG1 | 1.9 ± 0.39 | 7.9 ± 1.3 | 20 ± 6.9 |
| T204A | None | 0.082 ± 0.0061 | 31 ± 7.1 | 2.5 ± 0.40 |
| T204A | 0.1 µM DnaJB1 | 0.11 ± 0.020 | 27 ± 7.5 | 2.7 ± 0.37 |
| T204A | 0.3 µM DnaJB1 | 0.085 ± 0.098 | 38 ± 8.1 | 3.1 ± 0.35 |
| T204A | 1.0 µM DnaJB1 | 0.15 ± 0.036 | 26 ± 4.2 | 3.7 ± 0.17 |
| T204A | 0.1 µM BAG1 | 0.23 ± 0.041 | 12 ± 1.6 | 2.8 ± 0.11 |
| T204A | 0.3 µM BAG1 | 0.46 ± 0.10 | 9.0 ± 1.0 | 4.0 ± 0.42 |
| T204A | 1.0 µM BAG1 | 0.88 ± 0.064 | 4.7 ± 0.31 | 4.1 ± 0.24 |

1. Statistics on X-ray diffraction data collection and refinement data of the cryogenic Hsp72 crystals.

|  |  |  |
| --- | --- | --- |
|  | Y149A-ADP | Y149A-AMPPnP |
| Crystal data |  |  |
| Beamline | PF-17A | PF-17A |
| Temperature (K) | 100 | 100 |
| Resolution range (Å) | 47.69-1.80 (1.86-1.80) | 37.75-1.70 (1.76-1.70) |
| Space group | *P*212121 | *P*212121 |
| Unit-cell parameters (Å) | 45.9, 63.6, 144.1 | 45.8, 62.0, 142.8 |
| Observed reflections | 215672 (22121) | 200742 (18154) |
| Unique reflections | 39659 (3886) | 44823 (4381) |
| Completeness (%) | 99.1 (98.5) | 98.8 (97.6) |
| *R*meas (%) | 7.6 (70.1) | 7.4 (71.3) |
| *R*pim (%) | 3.2 (28.7) | 3.4 (33.7) |
| Redundancy | 5.4 (5.7) | 4.5 (4.1) |
| *I*/σ(*I*) | 16.5 (2.6) | 13.9 (2.5) |
| CC1/2 | 0.998 (0.878) | 0.998 (0.771) |
| Refinement data |  |  |
| *R*/*R*free (%) | 17.8/21.6 | 18.6/22.7 |
| RMSDbonds (Å) | 0.006 | 0.006 |
| RMSDangles (°) | 0.84 | 0.81 |
| Average *B*factor (Å2) |  |  |
| Overall/protein | 30.6/29.8 | 28/27.1 |
| Nucleotide/water/ion | 21.7/38/25.9 | 21.1/35.9/31.9 |
| Ramachandran plot (%) |  |  |
| Favored/allowed | 99.7/0.3 | 99.7/0.3 |
| PDB code | XXX2 | XXX3 |

Values for the outer shell are given in parentheses.

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Supporting information

1. Structural comparison of the NBD of Hsp72.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| RMSD (Å) | WT-ADP neutron @RT  (PDB code XXX1) | WT-AMPPnP @cryo  (PDB code 2E8A) | WT-ADP @cryo  (PDB code 3ATU) | Y149A-AMPPnP @cryo  (PDB code XXX2) | Y149A-ADP @cryo  (PDB code XXX3) |
| WT-ADP neutron @RT  (PDB code XXX1) | - | 0.39 | 0.28 | 0.38 | 0.38 |
| WT-AMPPnP @cryo  (PDB code 2E8A) |  | - | 0.37 | 0.34 | 0.35 |
| WT-ADP @cryo  (PDB code 3ATU) |  |  | - | 0.33 | 0.28 |
| Y149A-AMPPnP @cryo  (PDB code XXX2) |  |  |  | - | 0.25 |



1. Neutron and X-ray maps around the adenyl group and ribose moiety (a), and the phosphate moiety (b) (PDB code XXX1). The |*F*o|-|*F*c| Neutron scattering density map omitting deuterium atoms contoured at +4σ, and 2|*F*o|-|*F*c| electron density map omitting water molecules and sodium ions is contoured at 2σ.



1. Neutron and X-ray maps around the water cluster 2 (w12-w18) (a) and water cluster 3 (w19-w26) (b) (PDB code XXX1). The |*F*o|-|*F*c| Neutron scattering density map omitting deuterium atoms contoured at +4σ, and 2|*F*o|-|*F*c| electron density map omitting water molecules is contoured at 2σ.



1. X-ray crystal structure of Hsp72-AMPPnP (PDB code 2E8A) (a) and Hsp72-ADP (PDB code 3ATU) (b) superimposed on the neutron crystal structure of Hsp72-ADP (PDB code XXX1). The nucleotide molecules, Y204, Y149 of X-ray structure are shown in stick model. The water molecules of X-ray structure are shown in red sphere model. The water molecules of the overlaid neutron structure are shown in semitransparent stick model.



1. Omit difference Fourier maps for Y149A-Hsp72 in complex with ADP (PDB code XXX2) (a) and Y149A-Hsp72 in complex with AMPPnP (PDB code XXX3) (b). The electron density maps are contoured at 4σ. The side chain of Y149 and water cluster 3 of the superposed neutron structure (PDB code XXX1) are shown in semitransparent stick model.