Large-scale crystallization and neutron crystallographic analysis of HSP70 in complex with ADP

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HSP70 belongs to the heat-shock protein family and binds to unfolded proteins, driven by ATP hydrolysis, in order to prevent aggregation. Previous X-ray crystallographic analyses of HSP70 have shown that HSP70 binds to ADP with internal water molecules. In order to elucidate the role of the water molecules, including their H/D atoms, a neutron diffraction study of the human HSP70 ATPase domain was initiated. Deuterated large crystals of the HSP–ADP complex (1.2–1.8 mm³) were successfully grown by large-scale crystallization, and a neutron diffraction experiment at BIODIFF resulted in diffraction to a maximum resolution of 2.2 Å. After data reduction, the overall completeness, R_{meas} and average $I/\sigma(I)$ were 90.4%, 11.7% and 8.1, respectively, indicating that the data set was sufficient to visualize H and D atoms.

1. Introduction

Heat-shock proteins (HSPs) are highly conserved molecular chaperones found in various organisms from archaebacteria to humans (Hartl *et al.*, 2011). The HSPs form a group of protein families that include HSPA, HSPB, HSPC, HSPD and HSPH (Vos *et al.*, 2008). They assist in protein folding, disaggregation and membrane translocation by binding to substrate proteins. HSP70A (HSP70; alternatively HSP72) is a stress-inducible HSP member and is comprised of an N-terminal ATPase domain of 45 kDa, a linker region and a C-terminal peptide substrate-binding domain of 25 kDa. The basic function of HSP70s is to bind and release denatured polypeptide, which takes place in association with an ATP-hydrolysis reaction. The unfolded state of the peptide is stabilized, and controlled release allows progression along the folding pathway (Hartl, 1996). Thereby, HSP70 enables the cell to suppress the harmful aggregation of unfolded proteins during stress (Daugaard *et al.*, 2007).

During ATP hydrolysis, the conformation of HSP70 changes from the ATP-bound state to the ADP-bound state. The ATPase domain of HSP70 binds to ADP with high affinity (Arakawa *et al.*, 2011). In previous crystallographic studies, many water molecules were observed near the ADP-binding site (Sriram *et al.*, 1997). Such an internal hydration structure is likely to contribute to the function of HSP70, its conformational change and the stable binding of ADP (Schlessman *et al.*, 2008).

In addition, it has been shown that lowered expression of heat-shock cognate 71 kDa protein (HSC70) and HSP70

Table 1

Production information for HSP70 ATPase domain.

Source organism	<i>Homo sapiens</i>
DNA source	Synthesized
Expression vector	pET-22b(+)
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MKAAAIGIDLGTYSYCVGVFQHGKVEIIAN DQGNRTTPSYVAFDTERLIGDAKNQV ALNPQNTVFDKRLIGRKFQDPVQSDM KHWPFQVINDGDKPKVQVSYKGETKAFY PEEISSMVLTKMKEIAEAYLGYPVTNAV ITVPAYFNDSQRQATKDAGVIAGLNVLR IINEPTAAAIAYGLDRTGKGERNVLI LGGGTFDVSILTIDDGIFEVKATAGDTH LGGEDFDNRLVNHFEVEFKRKHKKDISQ NKRVRRLRTACERAKRTLSSSTQASLE IDSLFEGIDFYTSITRARFEELCSDLFR STLEPVEKALRDAKLDKAQIHDVLVGG STRIKVKQLLQDFNGRDLNKSINPDE AVAYGAAVQAAILLEHHHHHH

induces tumour-specific apoptosis but not in nontumorigenic cell lines (Powers *et al.*, 2008). These results indicate that HSP70 is a potential drug-target protein for an anticancer drug (Cheeseman *et al.*, 2016). If experimental information on the H atoms of the water and protein molecules could be made available, a precise pharmacophore model could be constructed based on the experimental data on hydrogen acceptors and donors. This in turn could lead to the design of a potent HSP70 inhibitor.

Because of this background, observation of the H atoms of water molecules and the protonation states of amino-acid residues is required to progress to the next research step. H atoms were not included in the crystal structure of HSP70, even when it was determined at the near-atomic resolution of 1.34 Å (PDB entry 5bn8; D. Narayanan, A. Pflug, T. Christopeit, P. Kyomuhendo & R. A. Engh, unpublished work). Since neutron protein crystallography (NPC) is available to observe H atoms at medium resolutions such as 2.0–2.5 Å (Niimura *et al.*, 1997; Yokoyama *et al.*, 2015; Casadei *et al.*, 2014; Huang *et al.*, 2014), it is appropriate to adopt NPC for the analysis of HSP70 in biochemical and medicinal studies.

Here, we report the successful large-scale crystallization of the HSP70 ATPase domain and neutron and X-ray diffraction data collection.

2. Materials and methods

2.1. Macromolecule production

The recombinant plasmid pET-22b(+)-HSPA1B ATPase domain (HSP70, residues 1–380) synthesized by GenScript was transformed into *Escherichia coli* BL21(DE3) cells (Table 1). HSP70A1A and HSP70A1B are collectively called HSP70 or HSP72 and share all but two (E110D and N499S) of their 641 amino acids (Vos *et al.*, 2008). The transformed cells were grown at 310 K with agitation at 250 rev min⁻¹ in Luria Broth medium containing 50 µg ml⁻¹ ampicillin for 2.5 h. Protein expression was then induced with 0.25 mM isopropyl β-D-1-thiogalactopyranoside and the cultures were incubated for 15 h at 293 K. The cells were subsequently harvested by

centrifugation at 4000 rev min⁻¹ for 10 min followed by resuspension in a lysis buffer consisting of 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 30 mM imidazole. The cells were lysed by sonication. The cell lysate was centrifuged at 9500g for 60 min. Ni–NTA affinity chromatography was performed and the fractions containing the desired protein were pooled and dialysed against a buffer consisting of 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The protein purity was confirmed by SDS–PAGE. The total yield was 16 mg protein per litre of cell culture. The protein solution was concentrated to 15 mg ml⁻¹ for crystallization experiments in H₂O buffer. For the preparation of deuterated protein, dilution with D₂O buffer (3.1 mM Tris, 16.9 mM Tris–HCl, 150 mM NaCl) and concentration was repeated three times, and consequently 98% of the H₂O buffer was replaced with D₂O buffer. The deuterated protein was concentrated to 9.8 mg ml⁻¹ and stored at 193 K.

2.2. Crystallization

Prior to crystallization, ADP and MgCl₂ were added to the protein sample. Initially, crystallization screening was performed with the screening kits JBScreen Classic 1, 2, 6 and 10 (Jena Bioscience) using the hanging-drop vapour-diffusion method at 293 K. Initial crystals were observed in a solution containing PEG 2000 MME or PEG 4000. As a result of optimization, shiny single crystals were obtained in a solution consisting of 20–25% PEG 2000 MME, 90 mM sodium acetate, 10 mM acetic acid, 200 mM MgCl₂, 10% (v/v) glycerol. Large crystals in H₂O (crystal 1) were obtained by sitting-drop vapour diffusion (Table 2). Visible crystals began to appear within two weeks and crystal growth stopped after two months. To achieve H/D exchange of crystal 1, the reservoir solution was replaced with D₂O crystallization buffer [25% PEG 2000 MME, 90 mM sodium acetate, 10 mM acetic acid, 0.2 M MgCl₂, 10% (v/v) glycerol] and incubated for three months. Large crystals in D₂O (crystal 2) were obtained using deuterated protein and D₂O crystallization buffer [16% PEG 2000 MME, 90 mM sodium acetate-d₄, 10 mM acetic acid-d₃, 200 mM MgCl₂, 10% (v/v) glycerol-d₈]. The pD value of the reservoir solution was 5.6. Crystals began to appear after two months and crystal growth stopped after four months. Prior to diffraction experiments, the crystals were mounted in quartz capillaries with reservoir solution to avoid dryness and sealed with beeswax and Capillary Wax (Hampton Research).

2.3. Data collection and processing

Neutron diffraction experiments were performed using the BIODIFF diffractometer at Forschungs-Neutronenquelle Heinz Maier-Leibnitz (FRM II, Germany), a monochromatic diffractometer with a neutron image-plate system (Heinz Maier-Leibnitz Zentrum, 2015). A full neutron data set was collected from crystal 2 at room temperature with a wavelength of 3.98 Å. The data set was collected with two crystal orientations. For the first orientation, 159 rotation frames were recorded with a rotation range of 0.3° per frame and an exposure time of 3180 s. For the second crystal orientation, 219 frames were collected with the same rotation range and

Table 2
Large-scale crystallization.

Crystal	1 (crystallized in H ₂ O and deuterated by vapour diffusion)	2 (crystallized in D ₂ O)
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	Falcon 60 × 15 mm dish	Corning 150 × 25 mm dish
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	13	9.4
Buffer composition of protein solution	20 mM Tris pH 8.0, 150 mM NaCl, 1.2 mM ADP, 10 mM MgCl ₂	3.1 mM Tris, 16.9 mM Tris-HCl, 150 mM NaCl, 1 mM ADP, 10 mM MgCl ₂
Composition of reservoir solution	20% PEG 2000 MME, 90 mM sodium acetate, 10 mM acetic acid, 200 mM MgCl ₂ , 10% (v/v) glycerol	16% PEG 2000 MME, 90 mM sodium acetate-d ₄ , 10 mM acetic acid-d ₃ , 200 mM MgCl ₂ , 10% (v/v) glycerol-d ₈
Volume and ratio of drop	400 µl, 1:1	1 ml, 1:1
Volume of reservoir (ml)	5	50
Volume and size of crystal	1.8 mm ³ , 3.5 × 1.3 × 0.8 mm	1.2 mm ³ , 3 × 1 × 1 mm

Table 3
X-ray and neutron data collection and processing.

Values in parentheses are for the outer shell.

	X-ray	Neutron
Diffraction source	AR-NW12A, PF	BIODIFF, FRM II
Wavelength (Å)	1.00	3.98
Temperature (K)	Room temperature	Room temperature
Detector	ADSC Quantum 270	Neutron image-plate (cylindrical)
Crystal-to-detector distance (mm)	150	200
Rotation range per image (°)	1	0.3
Total rotation range (°)	140	113.4
Exposure time per image (s)	1	3180
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	46.7, 64.6, 145.6	46.7, 64.6, 145.7
Resolution range (Å)	44.45–1.60 (1.66–1.60)	39.3–2.20 (2.26–2.20)
Total No. of reflections	327847 (32623)	47458 (3464)
No. of unique reflections	57639 (5579)	20952 (1673)
Completeness (%)	97.5 (95.4)	90.4 (88.5)
Multiplicity	5.7 (5.9)	2.3 (2.1)
$\langle I/\sigma(I) \rangle$	13.5 (2.4)	8.1 (2.9)
<i>R</i> _{meas}	0.081 (0.733)	0.117 (0.374)
<i>R</i> _{merge}	0.073 (0.668)	0.092 (0.281)
<i>R</i> _{p.i.m.}	0.034 (0.299)	0.070 (0.243)
Overall <i>B</i> factor from Wilson plot (Å ²)	22.6	23.3

exposure time. The collected data were indexed, integrated and scaled to a resolution ($d_{\min}$) of 2.2 Å using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The X-ray

diffraction data set was collected at room temperature on beamline AR-NW12A at the Photon Factory (PF), Japan. The crystal for X-ray diffraction was grown under a condition similar to that used for crystal 2, but the resulting crystal was much smaller than crystal 2 (<0.2 mm³). In order to adjust the beam intensity and reduce the radiation damage, the direct X-ray beam was attenuated to about 10%. The dose by the end of X-ray data collection was 0.02 MGy (Zeldin *et al.*, 2013). The X-ray diffraction data set was processed using the *XDS* program package (Kabsch, 2010). A summary of the X-ray and neutron crystallographic statistics is presented in Table 3.

3. Results and discussion

The human HSP70 ATPase domain was successfully expressed in an *E. coli* bacterial expression system, and a crystallizable protein sample was obtained through a single purification step using nickel-affinity chromatography (Table 1, Supplementary Fig. S1). For neutron diffraction experiments, two kinds of HSP70–ADP complex crystals were prepared (Fig. 1, Table 2). One was crystallized in H₂O crystallization buffer followed by deuterium exchange (crystal 1) and the other was crystallized in D₂O crystallization buffer using deuterated proteins and perdeuterated reagents (crystal 2). The initial neutron diffraction experiment at BIODIFF revealed that both crystals

Figure 1
Crystal photographs of crystal 1 (*a*) and crystal 2 (*b*). The scale bars represent 1 mm.

diffracted to 2.3 Å resolution (Fig. 2). Their diffraction qualities were likely to be similar, suggesting that H/D exchange of crystal 1 had been successfully achieved. Since crystal 2 had been grown using perdeuterated reagents, it was selected for full data collection. Although the initial diffraction experiments were performed at a wavelength of 3.4 Å, a longer wavelength of 3.98 Å was selected to avoid spot overlap when collecting the full data set. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 46.7$, $b = 64.6$, $c = 145.7$ Å, which are isomorphous with those of previously reported crystals (Wisniewska *et al.*, 2010; Arakawa *et al.*, 2011). The maximum resolution, overall completeness and R_{meas} were 2.2 Å, 90.4% and 11.7%, respectively, indicating that the data quality was sufficient for the visualization of H and D atoms (Table 3). To achieve joint X-ray and neutron refinement, X-ray diffraction data for the HSP70–ADP complex were also collected at room temperature (Supplementary Fig. S2). The crystals were grown under a crystallization condition similar to that used for crystal 2, but the resulting crystals were much smaller than crystal 2. The diffraction data were processed to 1.6 Å resolution, resulting in a completeness of 97.5% and an R_{meas} of 8.1%.

The present neutron crystallographic experiments for the HSP70 ATPase domain are the first for a member of the HSP70 and ATPase families. The optimal pH for the HSP70 ATPase activity has not been investigated. To date, kinetic assays have been carried out at neutral pH (Dores-Silva *et al.*, 2015). On the other hand, HSP70 crystal structures obtained at various pH values (5.5–7.5) have been reported. The overall structures at pH 5.5 (PDB entry 3atv) and at pH 7.5 (PDB entry 2e8a) (Arakawa *et al.*, 2011) were similar, with an r.m.s.d. value of 0.33 Å, and the water molecules around the ligand-binding site were conserved. These results suggest that the neutron crystal structure at pD 5.6 should be relevant for medicinal and biochemical studies. In particular, HSP70 has been indicated to be a potential therapeutic target of tumour cells (Powers *et al.*, 2008). The experimental information on H atoms will be important and helpful for the construction of a pharmacophore model in the course of *in silico* drug design. HSP70 is also known to bind to an ADP molecule with high affinity. The neutron structure will be helpful for revealing the relationship between the protonation state, the hydration and the high affinity of ADP. Joint X-ray/neutron refinement is currently in progress.

Figure 2
Neutron diffraction patterns of crystal 1 (a) and crystal 2 (b) measured at BIODIFF at a wavelength of 3.4 Å.

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