CORE
Metadata, citation and similar papers at core.ac.uk
Provided by ScholarWorks@UNIST

Acta Crystallographica Section F
Structural Biology Communications
ISSN 2053-230X

Crystallization and preliminary X-ray diffraction analysis of Trap1 complexed with Hsp90 inhibitors

Hanbin Jeong, Byoung Heon Kang and Changwook Lee*

Department of Biological Sciences, Ulsan National Institute of Science and Technology, 50 UNIST-gil, Eonyang-eup, Ulju-gun, Ulsan 689-798, Republic of Korea

Correspondence e-mail: changwook@unist.ac.kr

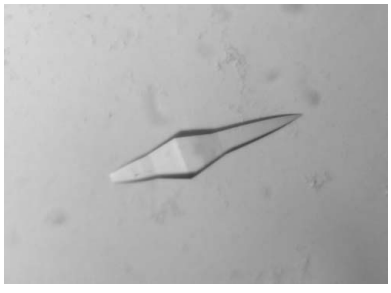
Received 16 October 2014
Accepted 13 November 2014

Hsp90 is a molecular chaperone responsible for the assembly and regulation of many cellular client proteins. In particular, Trap1, a mitochondrial Hsp90 homologue, plays a pivotal role in maintaining mitochondrial integrity, protecting against apoptosis in cancer cells. The N (N-terminal)-M (middle) domain of human Trap1 was crystallized in complex with Hsp90 inhibitors (PU-H71 and BIIB-021) by the hanging-drop vapour-diffusion method at pH 6.5 and 293 K using 15% PEG 8K as a precipitant. Diffraction data were collected from crystals of the Trap1–PU-H71 (2.7 Å) and Trap1–BIIB-021 (3.1 Å) complexes to high resolution at a synchrotron-radiation source. Preliminary X-ray diffraction analysis revealed that both crystals belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 69.2$, $c = 252.5$ Å, and contained one molecule per asymmetric unit according to Matthews coefficient calculations.

1. Introduction

Heat-shock protein 90 (Hsp90) is an essential molecular chaperone that has ATPase activity and regulates protein folding (Zhao *et al.*, 2005). Accumulating data have revealed that the client proteins for Hsp90 chaperones include cell-cycle proteins such as Cdc2, Cdk4, Cdk6 and Cdk9, signalling kinases such as HRI, Raf-1, Erb2 and Her2, and nuclear hormone receptors such as ER, AR and GR (Chiosis *et al.*, 2004; Pratt & Toft, 2003; Shao *et al.*, 2001). There are organelle-specific Hsp90 homologues, which include glucose-regulated protein 94 (Grp94, endoplasmic reticulum) and tumour necrosis factor receptor-associated protein 1 (Trap1, mitochondria), as well as a cytoplasmic Hsp90 (Chen *et al.*, 2005; Johnson, 2012; Taipale *et al.*, 2010). The architectures and molecular mechanisms of the ATPase activities of Hsp90 and Grp94 have been studied extensively by X-ray crystallography and biochemical experiments from bacteria to mammals (Ali *et al.*, 2006; Dollins *et al.*, 2007; Huai *et al.*, 2005; Pearl & Prodromou, 2006). The studies have elucidated that members of the Hsp90 family form a homodimer consisting of three distinct domains: an N-terminal ATP-binding domain (N-domain), a middle domain (M-domain) responsible for client-protein binding and catalytic reaction, and a C-terminal dimerization domain (C-domain). The structural studies also reveal a complex mechanism for ATPase-coupled conformational changes, which is critical for their chaperone activities (Krukenberg *et al.*, 2011).

Trap1 was first identified as a binding partner of tumour necrosis factor receptor 1 (TNFR1) using a yeast two-hybrid system (Song *et al.*, 1995). Human Trap1 contains 704 amino acids and shares structural and functional features with cytosolic Hsp90 and Grp94. Recently, crystal structures of ATP analogues bound to Trap1 from zebrafish have been solved and revealed that the asymmetric protomer organization of the Trap1 dimer plays a critical role in the ATPase cycle (Lavery *et al.*, 2014). Trap1 has emerged as a cancer therapeutic target because the activity of Trap1 has important implications for cytoprotection from DNA damage and apoptosis induced by oxidative stress conditions (Hua *et al.*, 2007; Kang, 2012; Montesano Gesualdi *et al.*, 2007; Kang *et al.*, 2009).



© 2014 International Union of Crystallography
All rights reserved

Table 1
Macromolecule-production information.

Source organism	*Homo sapiens*
DNA source	MGC full-length clone (Invitrogen)
Forward primer	5′-GCCGTAGGATCCAGCACGCAGACCGCCGAGGAC-3′
Reverse primers	
fTrap1	5′-GCCGTAATTGCGGCCGCTCAGTGTCGCTCCAGGGCCTT-3′
tTrap1	5′-GCCGGTCGACTCACTCCTTGTAGTGATCCACGAC-3′
Expression vector	pET-Duet
Expression host	*Escherichia coli* BL21(DE3)
UniProt accession No.	Q12931

To better understand the molecular mechanism of how Hsp90 ATPase inhibitors are specifically recognized by Trap1 and to design new inhibitors that exclusively target Trap1, it is essential to determine the three-dimensional structures of Trap1–inhibitor complexes. Here, we describe the purification and crystallization procedure of human Trap1 (N-M domain) complexed with the well known Hsp90 ATPase inhibitors PU-H71 and BIIB-021 and provide preliminary X-ray diffraction data.

Table 2
Data collection and processing.

Values in parentheses are for the highest resolution shell.

	tTrap1–PU-H71	tTrap1–BIIB-021
Beamline	5C, PAL	5C, PAL
Wavelength (Å)	0.97953	0.97953
Temperature (K)	100	100
Detector	ADSC Q315r	ADSC Q315r
Rotation range per image (°)	1	1
Total rotation range (°)	50	60
Exposure time per image (s)	1	1
Space group	$P4_12_12$ or $P4_32_12$	$P4_12_12$ or $P4_32_12$
a, b, c (Å)	69.22, 69.22, 252.52	69.46, 69.46, 252.81
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution range (Å)	35.0–2.70 (2.75–2.70)	35.0–3.10 (3.15–3.10)
Total No. of reflections	66641	53941
No. of unique reflections	17708 (864)	11899 (594)
Completeness (%)	99.4 (99.8)	98.5 (98.7)
Multiplicity	3.8 (3.9)	4.5 (4.7)
$\langle I/\sigma(I)\rangle$	24.5 (2.7)	20.1 (4.0)
$R_{\rm merge}$† (%)	7.3 (56.1)	10.0 (47.6)

† $R_{\rm merge} = 100 \times \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(hkl)$ for Miller indices hkl.

2. Materials and methods

2.1. Macromolecule production

For crystallographic studies, genes encoding full-length human Trap1 (residues 60–704, hereafter referred to as fTrap1) excluding the N-terminal mitochondrial targeting signal (residues 1–59) and a truncated version of Trap1 (residues 60–561, hereafter referred to as tTrap1) were amplified from an MGC full-length clone (Invitrogen) using PCR. The primers were forward, 5′-GCCGTAGGATCCAGCACGCAGACCGCCGAGGAC-3′, and reverse, 5′-GCCGTAATTGCGGCCGCTCAGTGTCGCTCCAGGGCCTT-3′ for fTrap1 and 5′-GCCGGTCGACTCACTCCTTGTAGTGATCCACGAC-3′ for tTrap1. The amplified gene was digested with the restriction enzymes *Bam*HI and *Not*I (704)/*Sal*I (561) at the N-terminus and C-terminus, respectively, and ligated into modified pET-Duet vector (Novagen) with an N-terminal histidine tag (6×His). This construct encoded a His_6-Trap1 protein with *Tobacco etch virus* (TEV) protease recognition sequences (ENLYGQS) between His_6 and Trap1 (Table 1).

The protein-expression and purification methods were the same for both fTrap1 and tTrap1. The plasmid encoding human His_6-Trap1 was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. A 5 ml seed of an overnight bacterial culture was transferred into 1000 ml fresh LB medium containing ampicillin (50 µg ml^{-1}) and grown at 310 K with vigorous shaking. When the cell density reached the mid-log phase (OD_{600} of 0.5–0.8), isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.2 m*M*. The cells were further cultured at 295 K for 15 h after induction to express the protein and were harvested by centrifugation at 10 000*g* for 15 min at 277 K. The pellet was resuspended in buffer *A* (25 m*M* sodium phosphate, 400 m*M* sodium chloride pH 7.4) plus 0.5 m*M* phenylmethylsulfonyl fluoride. The purification procedure comprised three consecutive chromatography steps including affinity, ion-exchange and size-exclusion chromatography. The cells were lysed by sonication on ice and the lysate was clarified by centrifugation at 40 000*g* for 50 min at 277 K. After centrifugation, the supernatant was loaded onto an Ni-charged chelating column (HiTrap Chelating column, GE Healthcare) equilibrated with buffer *A*. After washing with buffer *B* (25 m*M* sodium phosphate, 400 m*M* sodium chloride, 50 m*M* imidazole pH 7.4), the bound Trap1 protein was eluted from the column using buffer *C* (25 m*M* sodium phosphate, 300 m*M* sodium chloride, 400 m*M* imidazole pH 7.4). The eluted protein was dialyzed against 25 m*M* Tris, 100 m*M* sodium chloride, 5 m*M* dithiothreitol (DTT) pH 8.5 overnight to remove imidazole. During dialysis, the N-terminal His_6 tag was removed with TEV protease. The dialyzed protein solution was diluted with buffer *D* (25 m*M* Tris, 5 m*M* DTT pH 8.5) to reduce the concentration of sodium chloride to 50 m*M* and applied onto a 5 ml HiTrap Q column (GE Healthcare) pre-equilibrated with buffer *D*. After washing with buffer *D* (five column volumes), the protein was eluted with a linear gradient of 0–100% buffer *E* (25 m*M* Tris, 1 *M* sodium chloride, 5 m*M* DTT pH 8.5) in 30 column volumes. The protein was further purified with a Superdex 200 HR 16/60 gel-filtration column (GE Healthcare) equilibrated with buffer *F* (25 m*M* Tris, 150 m*M* sodium chloride, 5 m*M* DTT pH 7.5). The eluted Trap1 protein was finally concentrated to 20 mg ml^{-1} using an Amicon Ultra-15 centrifugal filter (50 kDa molecular-weight cutoff, Millipore) and flash-frozen in liquid nitrogen for storage. All purification steps were carried out at 277 K and were monitored by SDS–PAGE.

2.2. Crystallization

For the crystallization of Trap1–inhibitor complexes, two Hsp90 inhibitors (PU-H71 and BIIB-021) were dissolved in dimethyl sulfoxide (DMSO, Sigma). Prior to crystallization experiments, the Trap1 (full-length and truncated) protein was mixed with inhibitor in a 1:2 molar ratio for 50 min on ice. To minimize the damage to the protein by DMSO, we diluted the protein solution with buffer *F*, resulting in a final concentration of 9 mg ml^{-1} dissolved in less than 3% DMSO. Initial hanging-drop vapour-diffusion crystallization screening was performed at both 277 and 293 K using a 24-well VDX crystallization plate (Hampton Research) and commercially available screening solutions including Crystal Screen, Crystal Screen 2, Grid Screen (Hampton Research) and Wizard (Emerald Bio). Crystallization drops were prepared by mixing 1 µl Trap1 protein complexed with inhibitors and 1 µl screen solution. Rod-shaped crystals of fTrap1–PU-H71 were initially obtained using well solution consisting of 1.7 *M* sodium potassium phosphate, 5 m*M* DTT pH 6.5. tTrap1–PU-H71 and tTrap1–BIIB-021 were crystallized in the same crystallization buffer comprising 16% polyethylene glycol (PEG) 8000,

100 m*M* sodium cacodylate, 5 m*M* DTT pH 6.5. The initial crystallization condition was optimized by varying the protein concentration, the precipitant concentration and the pH and by using Additive Screen (Hampton Research).

2.3. Data collection and processing

For X-ray diffraction studies, crystals were transferred to a cryoprotection solution comprising reservoir buffer plus 30% glycerol and flash-cooled in liquid nitrogen. X-ray data were collected from the cooled crystals on beamline 5C of Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using a Q315r CCD detector. X-ray diffraction data were processed with *HKL*-2000 (Otwinowski & Minor, 1997). Complete data sets for the tTrap1–PU-H71 and tTrap1–BIIB-021 complexes were collected to 2.7 and 3.1 Å resolutions, respectively. However, fTrap1–PUH71 diffracted too poorly to collect complete data. The data-collection statistics are summarized in Table 2.

3. Results and discussion

The organelle-specific Hsp90 homologue proteins such as cytosolic Hsp90 and ER-resident Grp94 have previously been crystallized and their structures have been determined (Ali *et al.*, 2006; Dollins *et al.*, 2007). To crystallize a Trap1 protein, which is a mitochondrial Hsp90 homologue, we constructed both the full-length (fTrap1; residues 60–704) and a truncated version (tTrap1; residues 60–561) of human Trap1. fTrap1 and tTrap1 were expressed as His_6-tagged proteins at the N-terminus and were purified to homogeneity using the same procedures. The purity of proteins at the final purification step was at least 95% as monitored by SDS–PAGE (Fig. 1). We could obtain 20 mg of pure protein per litre of bacterial culture broth. Before crystallization trials, the purified proteins were mixed with Hsp90 inhibitors including PU-H71 and BIIB-021, which are well known small molecules that specifically target the Hsp90 ATPase domain. Rod-shaped crystals of the fTrap1–inhibitor complexes were obtained in a crystallization condition consisting of 1.7 *M* sodium potassium phosphate, 0.1 *M* HEPES, 0.4 *M* potassium chloride pH 7.5 at 293 K

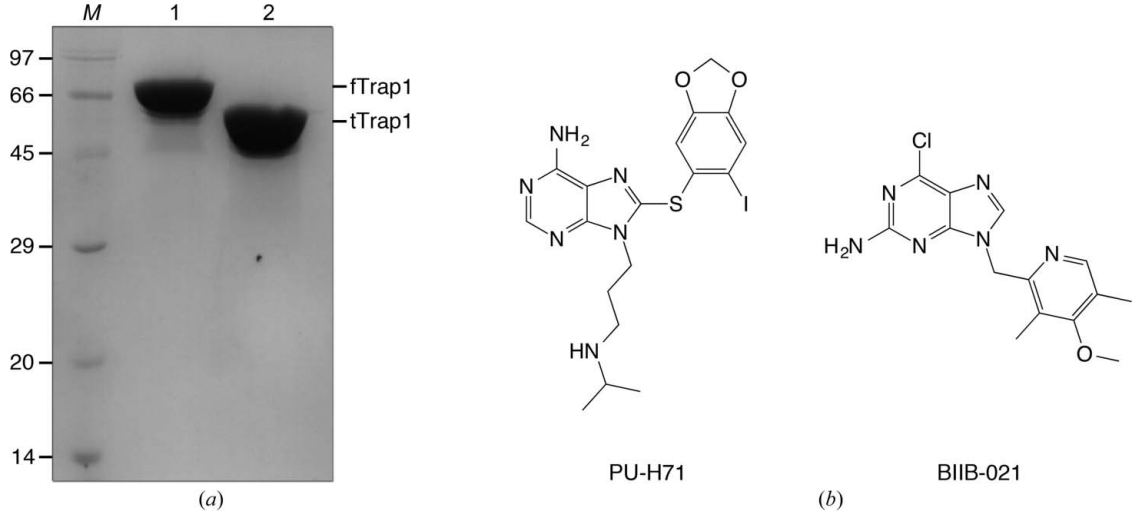


Figure 1
Proteins and inhibitors used in crystallization. (*a*) SDS–PAGE analysis showing purified human Trap1; lane 1, fTrap1 (residues 60–704); lane 2, tTrap1 (residues 60–561). Lane *M* contains molecular-weight marker (AccuLadder Protein Size Marker, Bioneer; labelled in kDa). (*b*) The purine-based Hsp90 inhibitors complexed with Trap1.

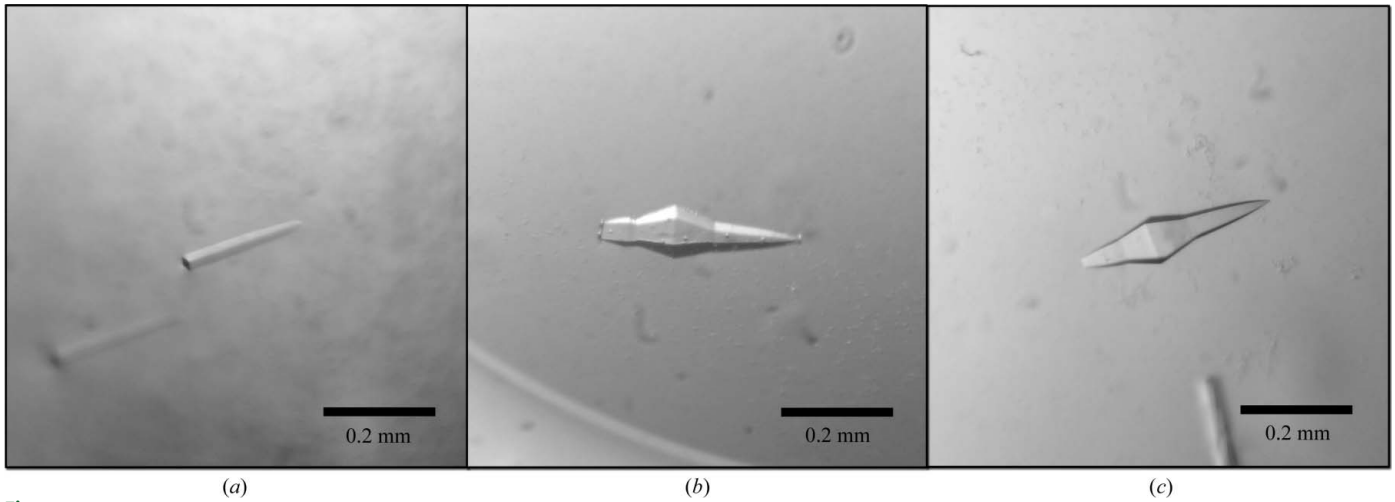


Figure 2
Crystals of human Trap1–inhibitor complexes. (*a*) Crystals of the human fTrap1–PU-H71 complex grown in 1.7 *M* sodium potassium phosphate, 0.1 *M* HEPES, 0.4 *M* potassium chloride pH 7.5 (maximum dimensions 0.02 × 0.02 × 0.2 mm). (*b*) Crystals of the human tTrap1–PU-H71 complex grown in 16% PEG 8K, 100 m*M* calcium acetate, 100 m*M* sodium cacodylate pH 6.5 (maximum dimensions 0.05 × 0.05 × 0.25 mm). (*c*) Crystals of the human tTrap1–BIIB-021 complex grown in the same condition as in (*b*).

by the hanging-drop vapour-diffusion method. The dimensions of the crystals were about 0.02 × 0.02 × 0.2 mm (Fig. 2*a*). The tTrap1–inhibitor mixture was crystallized in a reservoir buffer consisting of 16% PEG 8K, 0.1 *M* sodium cacodylate, 5 m*M* DTT pH 6.5 at 293 K. The initial condition was improved to give diffraction-quality crystals by adding 0.1 *M* calcium acetate and removing the reducing agent DTT (Fig. 2*b*). No difference in crystal shape was found between the PU-H71-bound and BIIB-021-bound crystals. From this observation, we propose that there is no significant structural difference between PU-H71–Trap1 and BIIB-021–Trap1. In order to collect X-ray diffraction data, we cryocooled crystals using cryoprotection solution consisting of reservoir solution plus 30% glycerol. We collected X-ray diffraction data on beamline 5C at PAL. While the crystals of the fTrap1–inhibitor complex diffracted to a maximum of ∼10 Å resolution, the tTrap1–inhibitor complex crystals displayed good-quality diffraction patterns. The crystals of PU-H71–tTrap1 and BIIB-021–tTrap1 diffracted to maximum resolutions of 2.7 and 3.1 Å, respectively (Fig. 3). The crystals belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 69.2$, $c = 252.5$ Å (Table 2). Assuming the presence of only one molecule per asymmetric unit, the Matthews coefficient (V_M) was estimated to be 2.64 Å^3 Da^{-1}, with a solvent content of 53% (Matthews, 1968). While we were attempting to solve the phase problem for the crystals, structures were determined of full-length zebrafish Trap1 in complex with ATP analogues (Lavery *et al.*, 2014). Human Trap1 has 74% sequence identity to zebrafish Trap1. We are currently trying to solve the structure of the

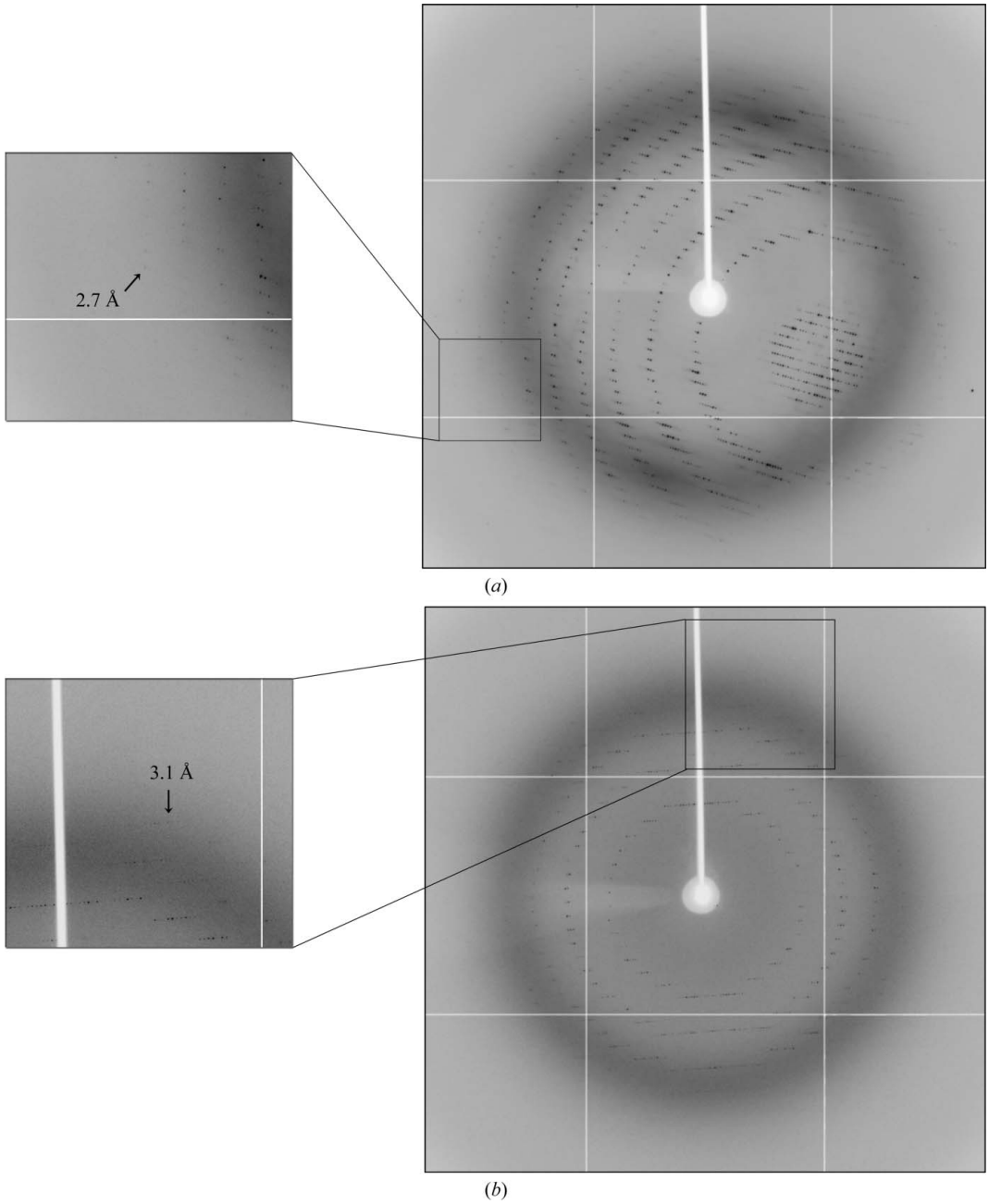


Figure 3
X-ray diffraction images. X-ray diffraction patterns collected from a single crystal of (*a*) the tTrap1–PU-H71 complex and (*b*) the tTrap1–BIIB-021 complex. The diffraction images were obtained using a synchrotron-radiation source. The maximum observed resolution for the tTrap–PU-H71 and tTrap1–BIIB-021 complexes is 2.7 and 3.1 Å, respectively.

human tTrap1–Hsp90 inhibitor complexes using molecular-replacement methods with the zebrafish Trap1 structure (PDB entry 4ivg; Lavery *et al.*, 2014) as a search model.

We thank the staff at beamline 5C of PAL for use of and assistance with the synchrotron facilities. This work was supported by the 2014 Research Fund (1.140023.01) of UNIST (Ulsan National Institute of Science and Technology). HJ was supported by the Global PhD Fellowship Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014H1A2A1020322).

References

Ali, M. M. U., Roe, S. M., Vaughan, C. K., Meyer, P., Panaretou, B., Piper, P. W., Prodromou, C. & Pearl, L. H. (2006). *Nature (London)*, **440**, 1013–1017.
Chen, B., Piel, W. H., Gui, L., Bruford, E. & Monteiro, A. (2005). *Genomics*, **86**, 627–637.
Chiosis, G., Vilenchik, M., Kim, J. & Solit, D. (2004). *Drug Discov. Today*, **9**, 881–888.
Dollins, D. E., Warren, J. J., Immormino, R. M. & Gewirth, D. T. (2007). *Mol. Cell*, **28**, 41–56.
Hua, G., Zhang, Q. & Fan, Z. (2007). *J. Biol. Chem.* **282**, 20553–20560.
Huai, Q., Wang, H., Liu, Y., Kim, H.-Y., Toft, D. & Ke, H. (2005). *Structure*, **13**, 579–590.
Johnson, J. L. (2012). *Biochim. Biophys. Acta*, **1823**, 607–613.
Kang, B. H. (2012). *BMB Rep.* **45**, 1–6.
Kang, B. H., Plescia, J., Song, H. Y., Meli, M., Colombo, G., Beebe, K., Scroggins, B., Neckers, L. & Altieri, D. C. (2009). *J. Clin. Invest.* **119**, 454–464.
Krukenberg, K. A., Street, T. O., Lavery, L. A. & Agard, D. A. (2011). *Q. Rev. Biophys.* **44**, 229–255.
Lavery, L. A., Partridge, J. R., Ramelot, T. A., Elnatan, D., Kennedy, M. A. & Agard, D. A. (2014). *Mol. Cell*, **53**, 330–343.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Montesano Gesualdi, N., Chirico, G., Pirozzi, G., Costantino, E., Landriscina, M. & Esposito, F. (2007). *Stress*, **10**, 342–350.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pearl, L. H. & Prodromou, C. (2006). *Annu. Rev. Biochem.* **75**, 271–294.
Pratt, W. B. & Toft, D. O. (2003). *Exp. Biol. Med. (Maywood)*, **228**, 111–133.
Shao, J., Grammatikakis, N., Scroggins, B. T., Uma, S., Huang, W., Chen, J.-J., Hartson, S. D. & Matts, R. L. (2001). *J. Biol. Chem.* **276**, 206–214.
Song, H. Y., Dunbar, J. D., Zhang, Y. X., Guo, D. & Donner, D. B. (1995). *J. Biol. Chem.* **270**, 3574–3581.
Taipale, M., Jarosz, D. F. & Lindquist, S. (2010). *Nature Rev. Mol. Cell Biol.* **11**, 515–528.
Zhao, R., Davey, M., Hsu, Y.-C., Kaplanek, P., Tong, A., Parsons, A. B., Krogan, N., Cagney, G., Mai, D., Greenblatt, J., Boone, C., Emili, A. & Houry, W. A. (2005). *Cell*, **120**, 715–727.