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Crystallization and preliminary neutron diffraction studies of ADP-ribose pyrophosphatase-I from *Thermus thermophilus* HB8

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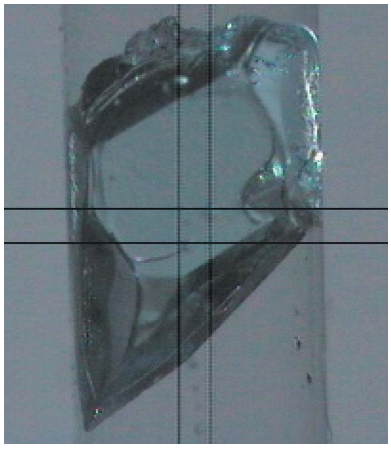


ADP-ribose pyrophosphatase-I from *Thermus thermophilus* HB8 (*Tt*ADPRase-I) prevents the intracellular accumulation of ADP-ribose by hydrolyzing it to AMP and ribose 5′-phosphate. To understand the catalytic mechanism of *Tt*ADPRase-I, it is necessary to investigate the role of glutamates and metal ions as well as the coordination of water molecules located at the active site. A macroseeding method was developed in order to obtain a large *Tt*ADPRase-I crystal which was suitable for a neutron diffraction study to provide structural information. Neutron and X-ray diffraction experiments were performed at room temperature using the same crystal. The crystal diffracted to 2.1 and 1.5 Å resolution in the neutron and X-ray diffraction experiments, respectively. The crystal belonged to the primitive space group $P3_221$, with unit-cell parameters $a = b = 50.7$, $c = 119$ Å.

1. Introduction

ADP-ribose pyrophosphatase (ADPRase) is one of the main Nudix proteins. Nudix proteins are widely distributed in nature (Bessman *et al.*, 1996; Lin *et al.*, 1997) and have a highly conserved amino-acid sequence motif referred to as the 'Nudix box' (GX_5EX_7REUXEEXGU, where U represents Ile, Leu or Val and X represents any amino acid), which forms a loop–helix–loop structure involved in catalysis (Gabelli *et al.*, 2001). Nudix proteins are 'housekeeping' enzymes, which obviate potentially toxic nucleotide metabolites from cells and control the cellular concentration of toxic nucleoside derivatives. The ADPRases prevent the intracellular accumulation of ADP-ribose (ADPR) by hydrolyzing it to AMP and ribose 5′-phosphate and are widely distributed in many organisms. The *Thermus thermophilus* HB8 genome has eight genes encoding Nudix proteins, designated Ndx1–Ndx8. Among these Nudix proteins, Ndx2 and Ndx4 are predicted to be ADPRases from their sequences. The differences in substrate specificity between Ndx2 and Ndx4 have been investigated by X-ray structural analysis (Yoshiba *et al.*, 2004; Ooga *et al.*, 2005; Wakamatsu *et al.*, 2008). According to Wakamatsu and coworkers, ADPRases can be classified into the categories ADPRase-I and ADPRase-II by their specificity for binding ADPR. The ADPRase-I proteins, including Ndx4, show a high specificity for ADPR, while the ADPRase-II proteins, including Ndx2, show a lower specificity for ADPR. In this study, we investigate an ADPRase-I (Ndx4) derived from *T. thermophilus* HB8 (*Tt*ADPRase-I). *Tt*ADPRase-I is highly specific for ADPR and catalyzes the hydrolysis of ADPR to AMP and ribose 5′-phosphate in the presence of Mg^{2+} and Zn^{2+} ions even at 348 K. In addition, *Tt*ADPRase-I is very stable towards heat, pH shift and denaturants (Yokoyama, Hirota *et al.*, 2000; Yokoyama, Matsui *et al.*, 2000), which is advantageous for investigation of the catalytic function of ADPRase.

It has been proposed that the ADPR hydrolysis pathway consists of four reaction states (Yoshiba *et al.*, 2004): bound with metal (I), with metal and substrate (II), with metal and substrate in the transition state (III) and with products (IV). It has also been proposed that coordination of both Glu82 and Glu86 to the metal ions is essential to activate the catalytic water molecule to cleave the nucleoside diphosphate linkage (Ooga *et al.*, 2005). To prove the

catalytic role of these glutamic acids using structural information on the H atoms and hydrating waters, we performed neutron crystallographic analysis of *Tt*ADPRase-I complexed with the nonhydrolyzable ADPR analogue α,β-methyleneadenosine diphosphoribose (AMPCPR). This approach had previously proved to be successful in the neutron structure analysis of the enzyme–inhibitor complex (Adachi *et al.*, 2004; Tamada *et al.*, 2009; Kuroki *et al.*, 2010). In this study, we report the purification, crystallization and preliminary neutron crystallographic analysis of *Tt*ADPRase-I.

2. Materials and methods

2.1. Expression and purification

*Tt*ADPRase-I was expressed and purified as described previously (Yoshiba *et al.*, 2003) with minor modifications. Briefly, the expression plasmid (pET-11a) containing the gene encoding *Tt*ADPRase-I was used to transform *Escherichia coli* strain BL21 (DE3) (Merck). The transformant was cultured at 310 K in Luria–Bertani medium containing 50 mg ml^{-1} ampicillin and *Tt*ADPRase-I was overproduced without adding IPTG.

The complete purification procedure was performed at room temperature. After the cultured cells had been suspended in 20 m*M* Tris–HCl pH 8.0 containing 5 m*M* β-mercaptoethanol and 50 m*M* NaCl, the cells were disrupted by sonication. The cell lysate was incubated at 343 K for 20 min, kept on ice and then centrifuged (12 000*g*) for 20 min at 277 K. Ammonium sulfate was added to the resulting supernatant to a final concentration of 1.35 *M*. The solution was applied onto a Toyopearl Phenyl-650M column (25 × 100 mm, Tosoh, Japan) equilibrated with 50 m*M* sodium phosphate pH 7.0 containing 1.35 *M* ammonium sulfate. The proteins were eluted with a linear gradient of 1.35–0 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.0. Fractions containing the target protein were collected, desalted by dialysis against 20 m*M* Tris–HCl pH 8.0 and applied onto a HiLoad 26/10 Q Sepharose HP column (GE Healthcare). Proteins were eluted with a linear gradient of 0–500 m*M* NaCl in 20 m*M* Tris–HCl pH 8.0. Fractions containing the *Tt*ADPRase-I protein were then dialyzed against 20 m*M* Tris–HCl buffer pH 8.0. The protein solution was concentrated to 75 mg ml^{-1} and stored at 277 K. 120 mg *Tt*ADPRase-I was finally purified from a 1 l culture.

2.2. Crystallization

The initial seed crystals were obtained by the sitting-drop vapour-diffusion method. The drops were prepared by mixing 20 µl reservoir solution with 20 µl 35 mg ml^{-1} *Tt*ADPRase-I in 20 m*M* Tris–HCl buffer pH 8.0 and were equilibrated against reservoir solution consisting of 18%(*w*/*v*) PEG 4000, 0.1 *M* sodium acetate buffer pH 5.3, 20%(*w*/*v*) glycerol, 0.2 *M* ammonium sulfate at 293 K.

Macroseeding was performed using seed crystals washed with solution *A* consisting of 18%(*w*/*v*) PEG 4000, 0.05 *M* sodium acetate buffer pH 5.3, 20%(*w*/*v*) glycerol, 0.2 *M* ammonium sulfate and then moved into 5 µl solution *B* [solution *A* but with 26%(*w*/*v*) PEG 4000 instead of 18%(*w*/*v*)] with a cryoloop. After keeping the washed seed crystals for 1 d in a 50 µl drop of solution *B*, 50 µl solution *A* containing 28 mg ml^{-1} protein was added to the drop. After 7 d, a further 200 µl of the same protein solution was added to the drop and left for 55 d to obtain a large crystal (2.5 × 2.5 × 1.5 mm). This process is shown in Fig. 1(*a*).

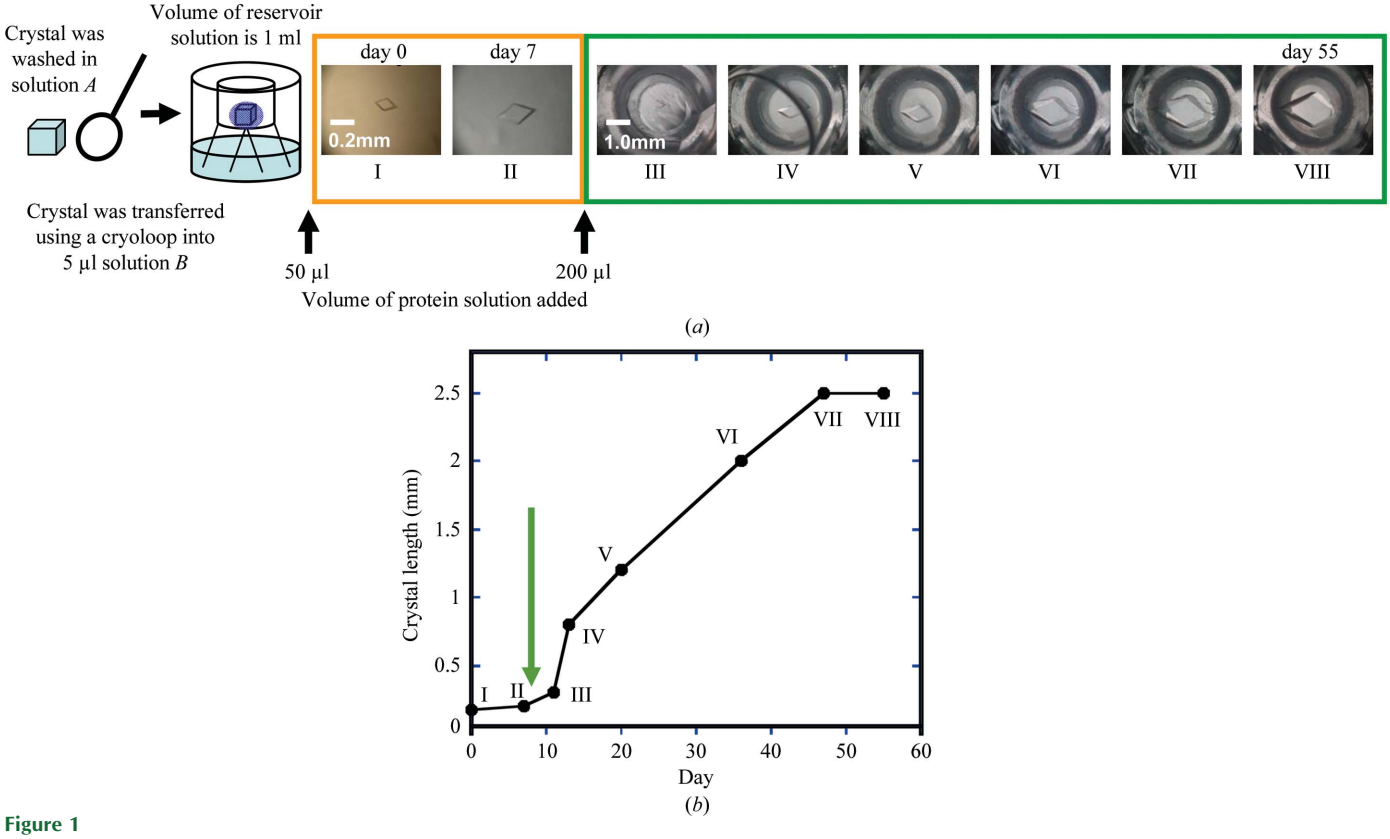


Figure 1
(*a*) Macroseeding procedure. The orange and green boxes contain pictures of the crystals after adding 50 and 200 µl of solution *A* containing protein, respectively. The roman numerals below the pictures correspond to those in (*b*). (*b*) Plot of crystal length *versus* elapsed time. The day on which 50 µl of solution *A* containing protein (28 mg ml^{-1}) was added is shown as day 0. 200 µl of the same protein solution was added at the point indicated by the green arrow (after 7 d).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Neutron	X-ray
Collected at	BIX-3, JRR-3	BL-6A, KEK-PF
Detector	Neutron imaging plate	Quantum 4R
Temperature (K)	293	293
Space group	$P3_221$	$P3_221$
Unit-cell parameters (Å)	$a = b = 50.7$, $c = 119$	$a = b = 50.7$, $c = 119$
Resolution range (Å)	43.9–2.10 (2.21–2.10)	29.5–1.50 (1.58–1.50)
No. of measured reflections	24909	303532
No. of unique reflections	8023	28885
Multiplicity	3.1 (2.4)	10.5 (10.7)
Mean $I/\sigma(I)$	18.6 (6.7)	41.0 (9.8)
$R_{\rm merge}$† (%)	9.5 (30.4)	3.7 (35.3)
$R_{\rm p.i.m.}$‡ (%)	5.7 (21.2)	1.2 (11.2)
Average mosaicity (°)	1.11	0.15
Completeness (%)	75.5 (62.8)	98.8 (99.8)
Wilson plot B factor (Å^2)	9.6	18.8

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of a reflection and $\langle I(hkl)\rangle$ is the average of the intensity. ‡ $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

Solvent exchange of the crystal prior to the neutron diffraction experiment was conducted by the vapour-diffusion method. The large crystal in 250 µl crystallization solution was vapour-diffused against 1000 µl 50 m*M* D-substituted sodium acetate buffer pD 5.3 containing 20%(*v*/*v*) D-substituted glycerol and 0.2 *M* $(ND_4)_2SO_4$ in D_2O for 7 d. The crystal was then transferred into 250 µl of the same buffer [50 m*M* D-substituted sodium acetate buffer pD 5.3 containing 20%(*v*/*v*) D-substituted glycerol and 0.2 *M* $(ND_4)_2SO_4$] and soaked for 21 d.

A crystal of the ternary complex consisting of *Tt*ADPRase-I, AMPCPR and Mg^{2+} was obtained by soaking the crystal in 50 m*M* D-substituted sodium acetate buffer pD 5.3 containing 20%(*v*/*v*) D-substituted glycerol, 0.2 *M* $(ND_4)_2SO_4$, 71 m*M* AMPCPR (a tenfold excess compared with *Tt*ADPRase-I) and 80 m*M* magnesium chloride.

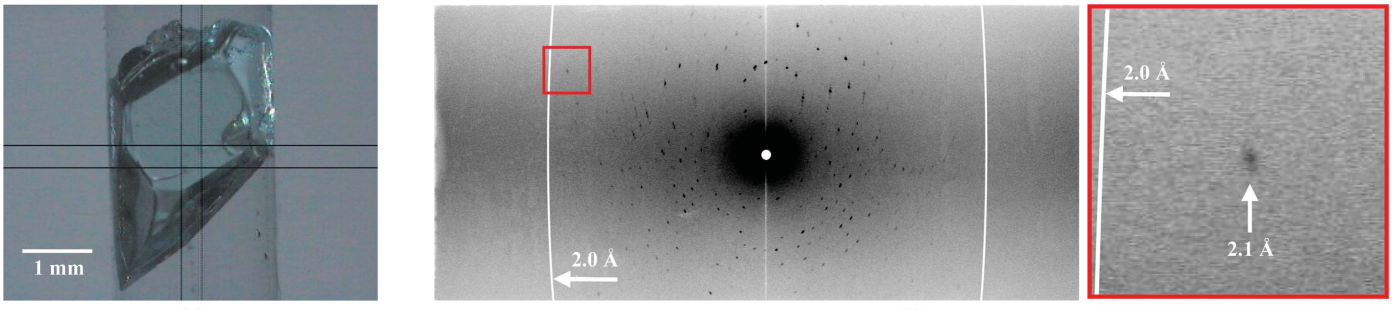
2.3. Neutron and X-ray diffraction

The crystal thus obtained was sealed in a quartz capillary (Fig. 2*a*) and used in both the neutron and X-ray diffraction experiments. The neutron diffraction data set was collected at room temperature with a monochromatic neutron beam (λ = 2.9 Å) on the BIX-3 diffractometer, which was installed at the 1G-A port of the JRR-3 research reactor of the Japan Atomic Energy Agency (Tanaka *et al.*, 2002), and diffraction signals were recorded on a neutron imaging plate. The diffraction data were collected to 2.1 Å resolution (Fig. 2*b*) using the step-scanning method and consisted of 285 still images covering 85.5° with an interval angle of 0.3°. The exposure was 4 h per frame. The neutron diffraction data from the ternary complex of *Tt*ADPRase-I with AMPCPR and Mg^{2+} were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997). Additional X-ray diffraction data were collected from the same crystal at room temperature on the BL-6A beamline of the Photon Factory (KEK, Japan) for joint refinement (Adams *et al.*, 2009). These data were processed using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997).

3. Results and discussion

A neutron diffraction experiment requires a relatively larger crystal than an X-ray diffraction experiment because of the weak neutron diffraction signal. Although the initial crystallization attempts using a 5 µl crystallization volume and the hanging-drop vapour-diffusion method yielded a *Tt*ADPRase-I crystal with dimensions of 1.0 × 1.0 × 0.4 mm, the crystal was still too small to obtain neutron diffraction signals using the BIX-3 diffractometer. Therefore, we attempted to grow the *Tt*ADPRase-I crystal further by increasing the crystallization volume in combination with the macroseeding procedure. The initial seed crystals were prepared by mixing a 20 µl reservoir of solution *A* with 20 µl protein solution containing 70 mg ml^{-1} *Tt*ADPRase-I. The resulting drops were equilibrated by vapour diffusion against a reservoir of solution *A*. After 30 d, initial crystals of 0.2 × 0.2 × 0.2 mm in size were obtained. The crystals with the clearest edges were selected and used as seeds.

After seeding, the growth of the crystal was monitored by measuring its length, as shown in Fig. 1(*b*). The labels I–VIII indicate the points at which the crystal length was observed and the corresponding crystal images are shown in Fig. 1(*a*). After the initial seeding into 50 µl of solution (the period from points I to II), a crystallization drop that did not contain a cracked crystal or a side crystal was chosen and 200 µl protein solution was added. After a slow growth phase (4 d from points II to III), followed by a short rapid phase (2 d from points III to IV), the crystal consistently grew to 2.5 × 2.5 × 1.5 mm (from points IV to VII). The crystal growth seemed to stop after 50 d; that is, at point VII. After 55 d, we harvested the crystal at point VIII; the crystal was 2.5 × 2.5 × 1.5 mm in size, which was appropriate for a neutron diffraction experiment. The crystal was then transferred into deuterated mother liquor before the neutron diffraction experiment in order to reduce the background noise caused by H atoms. The crystal was soaked in a solution (final pD 5.3) containing both AMPCPR and $MgCl_2$ for 2 d to obtain the ternary complex.



(*a*) (*b*)

Figure 2
(*a*) A *Tt*ADPRase-I crystal with approximate dimensions 2.5 × 2.5 × 1.5 mm (~10 mm^3) sealed into a quartz capillary (diameter of 2.5 mm). (*b*) An image of neutron diffraction spots taken from the *Tt*ADPRase-I crystal. The area in the small red box in the left-hand image is enlarged on the right.

Neutron data collection entailed 4 h of exposure for each image. These diffraction images were integrated and scaled into 8082 unique reflections with an R_{merge} of 9.7%. The crystal belonged to space group $P3_221$, with unit-cell parameters $a = b = 50.7$, $c = 119$ Å. The data-collection statistics are summarized in Table 1. The X-ray diffraction data set was collected using a Quantum 4R (Area Detector Systems Corporation, USA) charge-coupled device (CCD) installed on the BL-6A beamline of Photon Factory. The crystal diffracted to 1.5 Å resolution and belonged to space group $P3_221$, with unit-cell parameters $a = b = 50.7$, $c = 119$ Å at room temperature, as shown in Table 1. The neutron data set has sufficient quality to determine the locations of hydrogen and deuterium, considering their scattering lengths (Myles, 2006).

We also attempted to reduce the incoherent background noise originating from residual H atoms in *Tt*ADPRase-I. Because of the high yield of *Tt*ADPRase-I in this *E. coli* expression system, perdeuteration of *Tt*ADPRase-I using BioExpress cell-growth medium (U-D, 98%, Cambridge Isotope Laboratories) was performed and yielded 20 mg purified protein from a 1 l culture (data not shown). The perdeuteration of *Tt*ADPRase-I together with the reproducible preparation of large crystals will help us to examine the hydration structure of the active site of *Tt*ADPRase-I before substrate recognition and to elucidate the interaction of substrate or inhibitor with *Tt*ADPRase-I after soaking into the crystal. These neutron diffraction data will elucidate the catalytic mechanism of *Tt*ADPRase-I by permitting observation of the important water molecules and H atoms involved in catalysis.

The synchrotron-radiation experiment was performed on BL-6A at the Photon Factory (proposal No. 2008G075). We acknowledge the help provided by the beamline scientists at the Photon Factory. This work was supported in part by a MEXT Grant-in-Aid for Scientific Research (B) (22390010) (to RK).

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