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Overproduction, crystallization and preliminary diffraction data of ADP-ribose pyrophosphatase from *Thermus thermophilus* HB8

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An ADP-ribose pyrophosphatase from *Thermus thermophilus* HB8 was overproduced in *Escherichia coli* and purified. Gel-filtration chromatography showed the protein to be in a dimeric state. This protein catalyses the Mg^{2+}- or Zn^{2+}-dependent hydrolysis of ADP-ribose to AMP and ribose-5′-phosphate. It was crystallized in the absence and the presence of ADP-ribose by the hanging-drop vapour-diffusion method. Complete data sets were collected to 1.50 Å resolution from the apo form using synchrotron radiation and to 2.0 Å resolution from the complexed form. Both crystals belong to space group $P3_121$ or $P3_221$ and contain one molecule in the asymmetric unit.

1. Introduction

Nudix hydrolases catabolize nucleotide diphosphates linked to some other moiety, *x* (Bessman *et al.*, 1996), and have been observed in species ranging from prokaryotes to eukaryotes. This protein family is characterized by a signature sequence called the Nudix box ($GX_5EX_7REUXEEXGU$, where *U* is usually Ile, Val or Leu), representing a unique loop–helix–loop structure containing the catalytic and nucleotide-binding sites of the enzymes (Abeygunawardana *et al.*, 1995). Enzymes belonging to this family can be divided into subfamilies distinguished by their preference for nucleotide sugars or various ribo- or deoxyribonucleotides (Dunn *et al.*, 1999). These substrates include toxic nucleotide diphosphate derivatives, molecules involved in cell signalling processes and metabolic intermediates. Nudix hydrolases have been postulated to reduce the level of concentrations of these compounds in the cell (Safrany *et al.*, 1998; O'Handley *et al.*, 2001).

ADP-ribose, one of the nucleotide diphosphates, is a turnover product in the metabolisms of poly(ADP-ribose), cyclic ADP-ribose and NAD^+ metabolisms. High intracellular levels of ADP-ribose result in non-enzymatic ADP-ribosylation of proteins (McDonald & Moss, 1994), which inactivates these proteins and interferes with processes dependent on enzymatic ADP-ribosylation (Takada *et al.*, 1994). ADP-ribose pyrophosphatase (ADPRase), one of the ubiquitous Nudix enzymes present in all three kingdoms (Sheikh *et al.*, 1998), acts to prevent the intracellular accumulation of ADP-ribose by hydrolyzing ADP-ribose to AMP and ribose-5′-phosphate.

Proteins from *Thermus thermophilus* HB8 are well known for their stability towards heat, pH and denaturants as well as for their ease of crystallization (Nakagawa *et al.*, 1997; Sugahara *et al.*, 2000). Therefore, thermophilic proteins are well suited for structural and functional analyses. Genome analysis of *T. thermophilus* HB8 (Yokoyama *et al.*, 2000) suggested the presence of eight Nudix hydrolases including ADPRase. Accordingly, we performed crystallographic analysis of ADPRase from *T. thermophilus* HB8 (*Tt*ADPRase). Here, we report the purification, crystallization and preliminary crystallographic analysis of *Tt*ADPRase.

The crystal structure of *Escherichia coli* ADPRase (*Ec*ADPRase) has been solved at 1.9 Å resolution (Gabelli *et al.*, 2001, 2002). The *Tt*ADPRase crystal in this study gave high-resolution diffraction data to 1.50 Å.

2. Materials and methods

2.1. Cloning, sequencing and overproduction

We found a DNA sequence (DDBJ/EMBL/GeneBank AB107660; project code 1345) homologous to ADP-ribose pyrophosphatases in the *T. thermophilus* HB8 genome. Using this sequence information, we synthesized two primers for amplification of the target gene by polymerase chain reaction. Amplification was performed by standard protocols (Sambrook & Russel, 2001) and the amplified gene fragment was ligated into pT7Blue (Novagen) by TA cloning and confirmed by sequencing. Using the *Nde*I and *Bam*HI sites, the fragment bearing the target gene was ligated into pET-11a (Novagen). The resultant expression plasmid was used to transform *E. coli* strain BL21(DE3) (Novagen). The transformant was cultured at 310 K in Luria–Bertani medium containing ampicillin (50 μg ml^{-1}) and was overproduced without the addition of IPTG. Some expression of T7 RNA polymerase is

known to take place in the absence of IPTG (Studier, 1991). When target proteins are not toxic to *E. coli*, basal expression without IPTG induction sometimes leads to the production of a large amount of those proteins. In fact, many instances have been recorded of significant overproduction of *T. thermophilus* proteins in the absence of IPTG.

2.2. Protein purification

The complete purification procedure was conducted at room temperature. The cells (13 g) were suspended in 20 m*M* Tris–HCl pH 8.0, 5 m*M* 2-mercaptoethanol and 50 m*M* NaCl and then disrupted by sonication. The cell lysate was incubated at 343 K for 10 min, kept on ice and then ultracentrifuged (200 000*g*) for 1 h at 277 K. Ammonium sulfate was added to the resulting supernatant to a final concentration of 1.35 *M*. The solution was applied to a Resource ISO column (Amersham Biosciences) equilibrated with 50 m*M* sodium phosphate pH 7.0 containing 1.35 *M* ammonium sulfate and proteins were eluted with a linear gradient of ammonium sulfate from 1.35 to 0 *M* in 50 m*M* sodium phosphate. Fractions containing the target protein were collected, desalted with a HiPrep 26/10 desalting column (Amersham Biosciences) and applied in 20 m*M* Tris–HCl pH 8.0 to a Resource Q column (Amersham Biosciences). Proteins were eluted with a linear gradient of 0–500 m*M* NaCl in the same buffer. Fractions containing the target protein were then loaded onto a HiLoad 16/60 Superdex S-75 prep-grade column equilibrated with 20 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl and eluted with the same buffer. After desalting in 20 m*M* Tris–HCl pH 8.0, the fractions containing the target protein were concentrated and stored at 277 K. At each step, the fractions were analyzed by SDS–PAGE with 12.5%(*w*/*v*) acrylamide gel.

2.3. Characterization of the protein

The oligomeric state of *Tt*ADPRase in solution was determined by gel-filtration chromatography. *Tt*ADPRase was loaded onto a Superdex 75 HR 10/30 column with an ÄKTA explorer (Amersham Biosciences) and then eluted with 150 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0 at a flow rate of 0.8 ml min^{-1}. The calibration curve was obtained by the use of gel-filtration calibration kits of low molecular weight (Amersham Biosciences).

The activity of *Tt*ADPRase was measured by reversed-phase high-performance liquid chromatography. The reaction solution contained 20 m*M* Tris–HCl, 5 m*M* $MgCl_2$ or 250 µ*M* $ZnSO_4$, 0.1 *M* KCl, 1.2 n*M* *Tt*ADPRase and 200 µ*M* ADP-ribose at pH 7.5. The reaction mixture (50 µl) was incubated at 298 K for 10 min and the reaction was stopped by adding the same volume of 100 m*M* EDTA. The mixture was applied onto a C18 column (CAPCELL PAK C18 MG column, Shiseido) equilibrated with 20 m*M* sodium phosphate pH 7.0, 5 m*M* tetrabutylammonium phosphate and 5%(*v*/*v*) methanol with an ÄKTA explorer (Amersham Biosciences). The elution was performed at 1 ml min^{-1} with a 5–50% gradient of methanol in the equilibration buffer and the absorbance at 260 nm was monitored. AMP and the substrate were eluted at elution volumes of 5 and 10 ml, respectively. The amounts of compounds were assessed from their peak areas.

2.4. Crystallization

Crystallization conditions for *Tt*ADPRase were surveyed by the hanging-drop vapour-diffusion method with Crystal Screen Kits (Hampton Research) at 293 K. The initial protein concentration was 40 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 2 µl of the protein solution was mixed with the same volume of reservoir solution and equilibrated against the reservoir solution. Co-crystallization trials with ADP-ribose were set up in the same way, with the initial protein and ADP-ribose concentrations being 40 mg ml^{-1} and 2 m*M*, respectively.

2.5. X-ray diffraction analysis

The apo and complex crystals were mounted on a cryoloop and flash-cooled in a nitrogen-gas stream at 103 K. X-ray diffraction data for the apo form were collected with a MAR CCD using synchrotron radiation at BL44B2, SPring-8 (Adachi *et al.*, 2001). The oscillation angle was 0.8°, the exposure time was 10 s per frame and the camera distance was 100 mm. Diffraction images were processed using the *HKL*2000 program suite (Otwinowski & Minor, 1997). For the complex, the X-ray diffraction experiment was carried out in the laboratory using Cu *Kα* radiation and X-ray diffraction data were collected with an R-AXIS IV++ (Rigaku). The oscillation angle was 0.5°, the exposure time was 5 min per frame and the camera distance was 150 mm. Diffraction images were processed using the programs *MOSFLM* (Leslie, 1992) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

*Tt*ADPRase consists of 170 amino-acid residues and contains a characteristic Nudix box (**G**LIEPG**E**DPLEAAR**REL**A**EE**T**GL**). Its sequence shares a marginal sequence identity (28%) with that of *Ec*ADPRase (Fig. 1). We overproduced *Tt*ADPRase in *E. coli* and purified it to homogeneity as judged by SDS–PAGE. The final amount of purified protein was 133 mg and the yield was 10 mg of protein per gram of cells. On gel filtration, *Tt*ADPRase eluted at an elution volume corresponding to a molecular weight of about 38 000 Da, suggesting that *Tt*ADPRase exists as a dimer in solution. When *Tt*ADPRase reacted with ADP-ribose in the presence of Mg^{2+} or Zn^{2+}, a peak corresponding to that of AMP was obtained. These results indicate both that ADP-ribose was hydrolyzed to AMP and ribose-5′-phosphate and that Zn^{2+} activates as well as Mg^{2+}. In the absence of the divalent cations, the peak corresponding to AMP was not detected (data not shown).

Initial crystallization trials using Crystal Screen Kits yielded several crystal forms in the absence and the presence of ADP-ribose. Trigonal crystals were grown in a solution produced by diluting Crystal Screen No. 20 twofold with water. Their quality was

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Tt   1                          MGRVYYGGV- ERTYLYRGRI LN--LALEGR YEIVEHKPAV
Ec   1   MLKPDNLPVT FGKNDVEIIA RETLYRGFFS LDLYRFRHRL FNGQMSHEVR REIFERGHAA
                                    Y G       Y  R R   N     E R  EI E   A

Tt  38   AVIALREGR- -MLFVRQMR- ----PAVGLA PLEIPAGLIE PGEDPLEAAR RELAEETGLS
Ec  61   VLLPFDPVRD EVVLIEQIRI AAYDTSETPW LLEMVAGMIE EGESVEDVAR REAIEEAGLI
               R         Q R                LE  AG IE  GE     AR RE  EE GL

Tt  91   GDLTY-LFSY FVSPGFTDEK THVFLAE--- -NLKEVEAHP DEDEAIEVVW MRPEEALERH
Ec 121   VKRTKPVLSF LASPGGTSER SSIMVGEVDA TTASGIHGLA DENEDIRVHV VSREQAYQWV
           T    S    SPG T E        E                DE E I V      E A

Tt 146   QRGEVEFSAT GLVGVLYYHA FLRGR
Ec 181   EEGKID-NAA SVIALQWLQL HHQALKNEWA
           G     A
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Figure 1
Amino-acid sequence of the *T. thermophilus* (*Tt*) and *E. coli* (*Ec*) ADPRase. The sequence of *E. coli* was aligned with that of *T. thermophilus* using the *GENETYX-MAC* search homology program v. 10.1 (Software Development Co. Ltd, Tokyo). Sequence identities are indicated below the sequence.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.55–1.50 Å for the apo form and 2.12–2.01 Å for the complex).

	Apo form	Complex with ADP-ribose
Space group	$P3_121$ or $P3_221$	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 49.6$, $c = 117.9$	$a = b = 49.8$, $c = 119.3$
Resolution (Å)	50–1.50	19–2.01
No. of observations	408369	164512
Unique reflections	27765	11986
Data completeness (%)	97.2 (93.5)	99.9 (99.9)
Mean $I/\sigma(I)$	34.3 (3.60)	11.2 (10.3)
$R_{\rm merge}$† (%)	3.3 (25)	4.0 (5.7)

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j I_j(hkl)$, where $I_j(hkl)$ is the intensity of measurement j and $\langle I(hkl)\rangle$ is the mean intensity of multiple $I_j(hkl)$ observations for symmetry-related reflections.

further improved by varying the concentration of polyethylene glycol 4000 and the pH value. The best crystallization conditions were as follows: 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 5.0, 20% glycerol and 18%(*w*/*v*) polyethylene glycol 4000. The crystal belongs to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 49.6$, $c = 117.9$ Å. The crystals diffracted X-rays to better than 1.5 Å resolution using synchrotron radiation. Assuming the asymmetric unit to contain one subunit, the respective $V_{\rm M}$ and $V_{\rm sol}$ values (Matthews, 1968) are 2.2 Å^3 Da^{-1} and 0.44, indicating that the twofold axis of the dimeric protein coincides with the crystallographic axis. When ADP-ribose was present in the drop, a crystal was grown with Crystal Screen Cryo No. 49 [0.8 *M* lithium sulfate, 1.6%(*w*/*v*) polyethylene glycol 8000 and 20% glycerol]. This crystal diffracted X-rays to 2.0 Å resolution. Results of the preliminary X-ray intensity measurements are given in Table 1. Although *Ec*ADPRase (PDB codes 1g0s, 1g9q, 1khz and 1ga7) was used as a search model in molecular replacement with the *AMoRe* program (Navaza, 1994), the structure could not be solved, probably because of low amino-acid sequence identity (28%). In the crystal structures, the *Ec*ADPRase homodimer comprising two domains undergoes a conformational change upon substrate binding. It is known that such conformational changes as well as the orientation of the domains can differ between proteins that are homologous to each other (Ura *et al.*, 2001). In order to solve the structure by the MAD technique with selenium as the anomalous scattering atom, preparation of the selenomethionine-labelled protein and its crystallization are in progress.

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