crystallization communications

Preparation, crystallization and preliminary X-ray crystallographic studies of diadenosine tetraphosphate hydrolase from *Shigella flexneri* 2a

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Diadenosine tetraphosphate (Ap_4A) hydrolase (EC 3.6.1.41) hydrolyzes Ap_4A symmetrically in prokaryotes. It plays a potential role in organisms by regulating the concentration of Ap_4A *in vivo*. To date, no three-dimensional structures of proteins with significant sequence homology to this protein have been determined. The 31.3 kDa Ap_4A hydrolase from *Shigella flexneri* 2a has been cloned, expressed and purified using an *Escherichia coli* expression system. Crystals of Ap_4A hydrolase have been obtained by the hanging-drop technique at 291 K using PEG 550 MME as precipitant. Ap_4A hydrolase crystals diffract X-rays to 3.26 Å and belong to space group $P2_1$, with unit-cell parameters $a = 118.9$, $b = 54.6$, $c = 128.5$ Å, $\beta = 95.7°$.

1. Introduction

Diadenosine tetraphosphates (Ap4A) were discovered by P. Zamecnik in the mid-1960s. They were found to exist in the whole spectrum of organisms from bacteria to higher eukaryotes. Ap4A is a product of the reverse reaction of some aminoacyl-tRNA synthetases activating amino acids in bacteria, fungi, mammals and higher plants. In prokaryotes, Ap4A is considered to be an alarm signal as it plays roles in cell proliferation (Nishimura *et al.*, 1997; Nishimura, 1998), heat-shock and oxidative stress (Lee *et al.*, 1983).

The degradation of Ap4A is catalyzed by the following enzymes: (i) asymmetrically by Ap4A hydrolase (EC 3.6.1.17), mainly in higher eukaryotes, (ii) symmetrically by Ap4A hydrolase (EC 3.6.1.41), mainly in bacteria, and (iii) by dinucleoside polyphosphate phosphorylase (EC 2.7.7.53), mainly in lower eukaryotes (Guranowski, 2000).

Since the early 1980s, symmetrical Ap4A hydrolases have been discovered in many organisms, typically in bacteria (Guranowski, 2000). They catalyze the hydrolysis of Ap4A into two ADP molecules. In addition to the specific substrate Ap4A, they also hydrolyze Ap5A, Gp4G and other extended compounds. In all these reactions, one of the two products is the corresponding nucleoside diphosphate. The enzyme is important in responding to heat-shock and oxidative stress *via* regulating the concentration of Ap4A. Much research has pursued the functions of symmetrical Ap_4A hydrolases by using the *Escherichia coli apaH⁻* mutant, which showed a ≥16-fold increase in the basal level of Ap_4A at 303 K (Farr *et al.*, 1989). The mutant affected cell division (Nishimura, 1998) and the expression level of some genes regulated by cAMP-binding protein and cAMP (Farr *et al.*, 1989) because of the accumulation of Ap_4A. A hypothesis has been made about the mechanism of the hydrolyzing reaction based on the possibility of substrate stereochemistry (Guranowski *et al.*, 1994). However, no structural information has yet been obtained to prove this hypothesis.

The asymmetrical Ap_4A hydrolase and the symmetrical enzyme both play a key role in regulating the intracellular Ap_4A level and hence potentially the cellular response to metabolic stress, but these two reactions have different products. Structures of asymmetrical Ap_4A hydrolases from various organisms show that this enzyme binds and locates its substrate to the catalytic machinery of the Nudix motif (Bailey *et al.*, 2002; Fletcher *et al.*, 2002). However, according to a putative conserved-domain prediction (*BLAST* online), symmetrical Ap_4A hydrolases do not have conservative primary- and
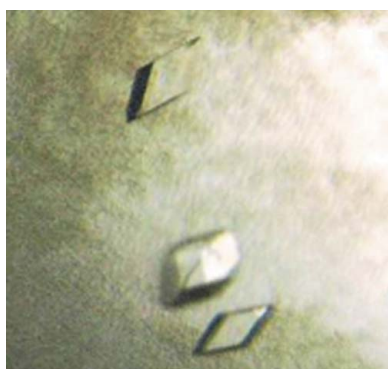
Table 1
X-ray diffraction and data-collection statistics.

Values in parentheses are for the outermost shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 118.9$, $b = 54.6$, $c = 128.5$, $\beta = 95.7$
Wavelength (Å)	1.0
Resolution range (Å)	20.0–3.26 (3.38–3.26)
No. of observed reflections	79937
No. of unique reflections	26171
$\langle I/\sigma(I)\rangle$	8.42 (3.19)
Completeness (%)	99.4 (99.4)
R_{merge}† (%)	13.9 (37.8)

† $R_{\text{merge}} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

secondary-structural similarity to the Nudix family. This means that the enzymes from bacteria and mammals have different mechanisms of binding and hydrolyzing Ap$_4$A. Thus, understanding of the structure, mechanism and substrate specificity of Ap$_4$A hydrolase in bacteria will be very helpful in designing Ap$_4$A analogues as potential therapeutic agents.

Here, we report the preparation and preliminary crystallographic studies of the Ap4A hydrolase from *Shigella flexneri* 2a which may cause bacillary dysentery or shigellosis in human.

2. Cloning of Ap$_4$A hydrolase gene from *S. flexneri* 2a

Cloning of the Ap$_4$A hydrolase gene (*apaH*) took place using the forward primer 5′-GGATTCCATATGGCGACATACCTTATTGGC 3′ and the reverse primer 5′-GGAATCCTCGAGAGACGCCGC-CGCTTC-3′. These primers introduced *Nde*I and *Xho*I restriction sites. The PCR used an *S. flexneri* 2a (strain 301) genomic DNA template. The PCR DNA fragment digested with the *Nde*I/*Xho*I enzymes was ligated to the vector pET-22b(+) digested with the same enzymes to obtain pET-22b-Ap$_4$A hydrolase. The plasmid was sequenced to confirm the insertion of the gene.

3. Expression and purification of Ap$_4$A hydrolase from *S. flexneri* 2a

A 1 l culture of *E. coli* BL21(DE3) cells harbouring pET-22b-Ap$_4$A hydrolase was grown at 310 K in a shaking flask with LB medium. When the culture reached $A_{600\,\text{nm}} = 0.5$–0.6, the *lac* promoter was induced with 0.8 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h induction, cells were harvested by centrifugation.

Cell pellets were resuspended in 30 ml buffer *A* (25 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10% glycerol). After sonication, soluble and insoluble fractions were separated by centrifugation. A 3 ml Ni-chelating Sepharose column was washed with 20 ml buffer *A* and the supernatant was applied onto the column and eluted with buffer *B* (25 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl) containing 20, 50, 100 and 150 m*M* imidazole in turn. The fractions eluted with buffer *B* containing 100 m*M* imidazole were collected and applied onto a 20 ml Resource-Q anion-exchange column (Amersham Pharmacia Biotech). The column was washed with 500 ml 25 m*M* Tris–HCl pH 8.0, 3 m*M* DTT (dithiothreitol) and developed with 25 m*M* Tris–HCl pH 8.0, 3 m*M* DTT containing 1 *M* NaCl. The fraction containing the enzyme was concentrated to 1 ml and was applied onto a HiLoad 16/60 Superdex G75 column (prep grade; Amersham Pharmacia Biotech), which was washed with buffer consisting of 10 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 3 m*M* DTT. After concentrating the fraction containing the enzyme, we obtained protein solution at a concentration of 8 mg ml⁻¹ in 10 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 3 m*M* DTT buffer. The protein solution was frozen in liquid nitrogen and stored at 200 K.

4. Character of the recombinant protein

The size of the recombinant protein was determined by SDS–PAGE and a band was located at around 32 kDa corresponding to the recombinant protein with a His tag. The oligomerization state of the protein in buffer was examined using DynaPro dynamic light scattering (Protein Solutions Co.) and the data were analyzed using *DYNAMICS* v.5.26.39. The results showed that the protein formed a dimer in the buffer and did not aggregate. After a month, the reserved recombinant protein was examined again and led to the same result of a dimer in the buffer and no aggregation. This shows that the protein was stable in the buffer under the storage conditions.

5. Crystallization, X-ray data collection and processing

Ap$_4$A hydrolase was crystallized by the hanging-drop vapour-diffusion method using Linbro plates at 291 K. 1 µl protein solution with concentration of about 8 mg ml⁻¹ was typically mixed with 1 µl reservoir solution and suspended on a cover slip over 0.4 ml reservoir solution. The protein was initially screened with Crystal Screens I and II and Index Screen (Hampton Research). Initial microcrystals appeared after only 1 d in reservoir solution consisting of 0.2 mol l⁻¹ magnesium formate, 12.5% PEG 3350. Using streak-seeding, single crystals were obtained 5 d later in reservoir solution consisting of 0.05 mol l⁻¹ MgCl$_2$, 0.1 mol l⁻¹ HEPES pH 7.5, 28% PEG 550 MME (Fig. 1) and had typical dimensions of 0.20 × 0.15 × 0.08 mm. The crystals were picked up in Hampton mounting loops and frozen directly in liquid nitrogen without cryobuffer.

A data set was collected from the protein crystals at the Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences using beamline 3W1A at 100 K with a MAR 165 mm CCD area detector. All diffraction data were indexed, integrated and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996). The X-ray diffraction parameters and the data-collection statistics are listed in Table 1.
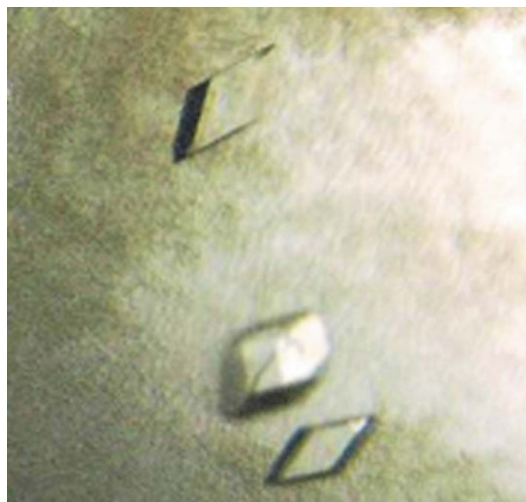
Figure 1
Crystals of Ap$_4$A hydrolase from *S. flexneri* 2a.

6. Discussion

The Ap$_4$A hydrolase from *S. flexneri* 2a has been prepared and crystallized successfully. Although the Ap$_4$A hydrolase crystal was sufficiently large in size, the crystals only diffracted to 3.26 Å. This may be a consequence of the crystal quality and possible damage by the synchrotron radiation.

Several studies have demonstrated that the primary and secondary structure of a partial sequence of Ap$_4$A hydrolase in bacteria is similar to that of serine/threonine phosphatases in eukaryotes. There are three conservative consequences including several conservative residues and two of them form a motif (Barton *et al.*, 1994). Although the crystal structures of various types of serine/threonine phosphatase have been determined, the sequence similarity between Ap$_4$A hydrolase and the phosphatases is low. Therefore, selenomethionine-substituted Ap$_4$A hydrolase is being prepared and crystallized. We hope that the structure of Ap$_4$A hydrolase will soon be determined and will make an important contribution to understanding the structure–function relationship of this type of enzyme.

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