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STRUCTURE NOTE

Crystal Structure of the Diadenosine Tetraphosphate Hydrolase From *Shigella flexneri 2a*

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Introduction. Diadenosine tetraphosphate (Ap_4A) was discovered by Zamecnik in the middle 1960s.[1] Ap_4A is a product of the back reaction of the amino acid activation catalyzed by some aminoacyl-tRNA synthetases.[2] It is widely agreed that there are aspects about the function of Ap_4A. On the one hand, it may be an unavoidable by-product of protein synthesis that has to be cleared from the cell before it attains a potentially toxic concentration. On the other hand, Ap_4A can function as a signal molecule and be deeply involved in regulation of DNA replication, cell division, and stress response.[3]

There are three kinds of Ap_4A hydrolases, that is, asymmetrically cleaving Ap_4A hydrolase (EC 3.6.1.17), symmetrically cleaving Ap_4A hydrolase (EC 3.6.1.41), and dinucleoside polyphosphate phosphorylase (EC 2.7.7.53). Asymmetrical Ap_4A hydrolases mainly exist in higher eukaryotes and play a key role in regulating the intracellular Ap4A level. Symmetrical Ap_4A hydrolases have been isolated from many organisms, typically from bacteria. It catalyses the hydrolysis of Ap_4A into two ADP and also hydrolyzes Ap_5A, Gp_4G, and other extending compounds. Furthermore, it is important in responding to heat shock and oxidative stress via regulating the concentration of Ap_4A in bacteria.[4]

Here we report the crystal structure of the symmetrically cleaving Ap_4A hydrolase from *Shigella flexneri 2a* which may cause bacillary dysentery or shigellosis in man.

Materials and Methods. *Cloning, Expression, and Purification.* The gene of Ap_4A hydrolase from *Shigella flexneri 2a str. 301* (Genbank: NC_004337) was cloned into the pET-22b(+) expression vector (Novagen). SeMet-substituted proteins were produced in *E. coli* BL21 (DE3). After harvested by centrifugation, the cells were resuspended in 30 mL of lysis buffer (25 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 10% glycerol, 5 m*M* β-Mercaptoethanol) and sonicated. The cell lysate was centrifugated at 12,000 rpm at 4°C for 30 min. Then the soluble fraction was applied to a Ni-NTA column (Amersham Pharmacia Biotech) preequilibrated with lysis buffer. The column was washed with wash buffer (25 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 20 m*M* imidazole, 5 m*M* β-Mercaptoethanol). Finally the target protein was eluted with elution buffer (25 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 200 m*M* imidazole, and 5 m*M* β-Mercaptoethanol). The eluate was applied to a 1-mL RESOURCE-Q anion exchange column (Amersham Pharmacia Biotech). The protein was eluted by using a linear gradient of 0–0.6*M* NaCl. The fraction with the target protein was concentrated to 1 mL, and was applied to HiLoad™ 16/60 Superdex 75 (prep grade) (Amersham Pharmacia Biotech), which was eluted with buffer consisting of 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, and 5 m*M* DTT.

Crystallization and Data Collection. Crystals of SeMet-substituted Ap_4A hydrolase were grown at 291 K by the hanging-drop vapor diffusion method. The reservoir solution was 100 m*M* Bis-Tris buffer (pH 6.8), containing 200 m*M* Magnesium chloride and 25% polyethylene glycol 3350. Protein solution (1 μL) with concentration of about 5 mg/mL was typically mixed with 1 μL reservoir solution and suspended on a cover slip over 0.45 mL reservoir solution. The crystals were picked up with Hampton mounting loops and frozen directly in liquid nitrogen. The crystal belongs to the space group C2, with unit cell dimensions $a = 167.50$ Å, $b = 54.97$ Å, $c = 119.20$ Å, and $\beta = 129.14°$. There are two molecules per asymmetric unit.

Structure Solution and Refinement. X-ray diffraction data of the SeMet-substituted Ap_4A hydrolase crystal

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TABLE I. Data Collection, Phasing, and Refinement Statistics

Data collection	
Resolution(Å)	50–2.72 (2.82–2.72)
Space group	C_2
Wavelength (Å)	0.9793
Molecules/ASU	2
Unit cell parameters	$a = 167.50$ Å, $b = 54.97$ Å, $c = 119.20$ Å, $\beta = 129.14^\circ$
Total observations	149,957
Unique reflections	21,524
Completeness (%)	99.7 (99.4)
Mean I/σ	17 (5)
R_{sym}[a] **(%)**	8.4 (43.1)
Average redundancy	6.97 (3.8)
Phasing	
Number of Se sites/ASU	8
Resolution range of data used	38.95–2.8
FOM	0.25
FOM after resolve	0.69
Refinement	
Resolution limits	38.95–2.72
R_{cryst}/R_{free}[b]	22.8/26.5
Sigma cutoff (%)	0
Ramachandran stastics (%)	
Most favored regions	88.2
Additionally allowed regions	10.7
Generously allowed regions	1.1
Disallowed regions	0
Rms deviations	
Bond length (Å)	0.01
Bond angle (°)	1.56

The values in parentheses refer to data in the highest shell.
[a] $R_{sym} = \Sigma(|I| - |\langle I \rangle|)/\Sigma|I|$.
[b] R_{free} calculated using random 5% of total reflections omitted from refinement.

were obtained on the beamline 3W1A at the Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences, using a MAR 165 mm CCD area detector at 100 K. Diffraction data were processed with the programs DENZO and SCALEPACK.[5] The initial phasing was solved by SAD method[6] using the program SOLVE.[7] The initial model consisting of 30% of the Ap_4A hydrolase structure was built by the program RESOLVE.[8] The rest of the model was manually built using O.[9] Refinement was carried out using the program REFMAC.[10] The stereochemistry of the structure was checked by the program PROCHECK.[11] The final model comprises 534 amino acid residues, 162 water molecules, and four manganese ions in the asymmetric unit. 24 amino acid residues at the C-terminal were not located, due to lack of electron density, and were excluded from the final model in the asymmetric unit. The statistic is summarized in Table I.

ICP Optical Emission Spectrometry. Identification of the metal ion in the Ap_4A hydrolase was carried out with an ICP optical emission spectrometer (ICP-OES). Purified protein was applied for the ICP-OES with protein concentration of about 0.3 mg/mL.

Results and Discussion. The crystallographic asymmetric unit of the Ap_4A hydrolase structure contains two molecules (A and B) that are related by a noncrystallographic twofold axis. The structures of two molecules are very similar, with root-mean-square (rms) deviation of 0.46 Å for all α carbon atoms [Fig. 1(A)]. The overall structure of Ap_4A hydrolase monomer is shown in Figure 1(B). The Ap_4A hydrolase molecule has dimensions of approximately 50 Å × 35 Å × 30 Å. The secondary structure elements, including 12 α-helices and 9 β-strands, are shown in Figure 1(B). The determined structure shows the existence of a narrow groove along the molecule surface, which is composed of α1, α2, α3, and α12. Based on the Fo–Fc density map and the result of the ICP-OES measurement, two manganese(II) ions separated by a distance of 3.45 Å have been found at the bottom of the groove. The dinuclear manganese cluster is bound to Asp8, His10, Asp37, Asn65, His120, His227, and four water molecules in the groove [Fig. 1(C)]. The six residues participating in binding to the dimanganese cluster are highly conserved in the protein family (Fig. 2).

Recently, the crystal structure of asymmetrical Ap_4A hydrolase from *Caenothabditis elegans* (PDB ID 1KT9[1]) was determined.[14] Although symmetrical Ap_4A hydrolase and asymmetrical Ap_4A hydrolase share the same substrate Ap_4A, the structural comparison between them indicates that the structure of symmetrical Ap_4A hydrolase is not notably similar to that of asymmetrical Ap_4A hydrolase. Using the DALI server[15] (http://www.edi.ac.uk/dali/), the symmetrical Ap_4A hydrolase structure was compared with the structures in the PDB database. The comparison results have revealed that the closest structural homologue is the bacteriophage λ Ser/Thr protein phosphatase (λPP, PDB ID 1G5B). This is also consistent with the results of conservation analysis by Barton et al.[16] However, there is only 21% sequence identity between Ap_4A hydrolase and λPP. The major differences between the two structures are located at the C-terminus where Ap_4A hydrolase has six additional α-helices and an additional β-strand. λPP has a phosphatase active site with the sequence motif DXH(X)*n*GDXXDR(X)*n*-GNHE and a dinuclear metal cluster.[17] Most of the sequence motif and the dinuclear metal cluster are also present in symmetrical Ap_4A hydrolase and homolog proteins [Figs. 1(C) and 2]. The structural comparison shows that Ap_4A hydrolase has a spacial residue arrangement similar to the structure of λPP active site. The putative active site of symmetrical Ap_4A hydrolase may resemble the λPP active site.

The crystal structure of the Ap_4A hydrolase from *Shigella flexneri* 2*a* is the first structure determined for symmetrical Ap_4A hydrolases. Its structure, in combination with further biochemical and biophysical studies, may reveal the structure–function relationship of symmetrically cleaving Ap_4A hydrolases.

Acknowledgments. Diffraction data were collected at Beijing Synchrotron Radiation Facility (BSRF).

[1]Protein Data Bank Accession Code. Coordinates and structure factors for the structure of Ap_4A hydrolase have been deposited at the Protein Data Bank with ID code 2DFJ.

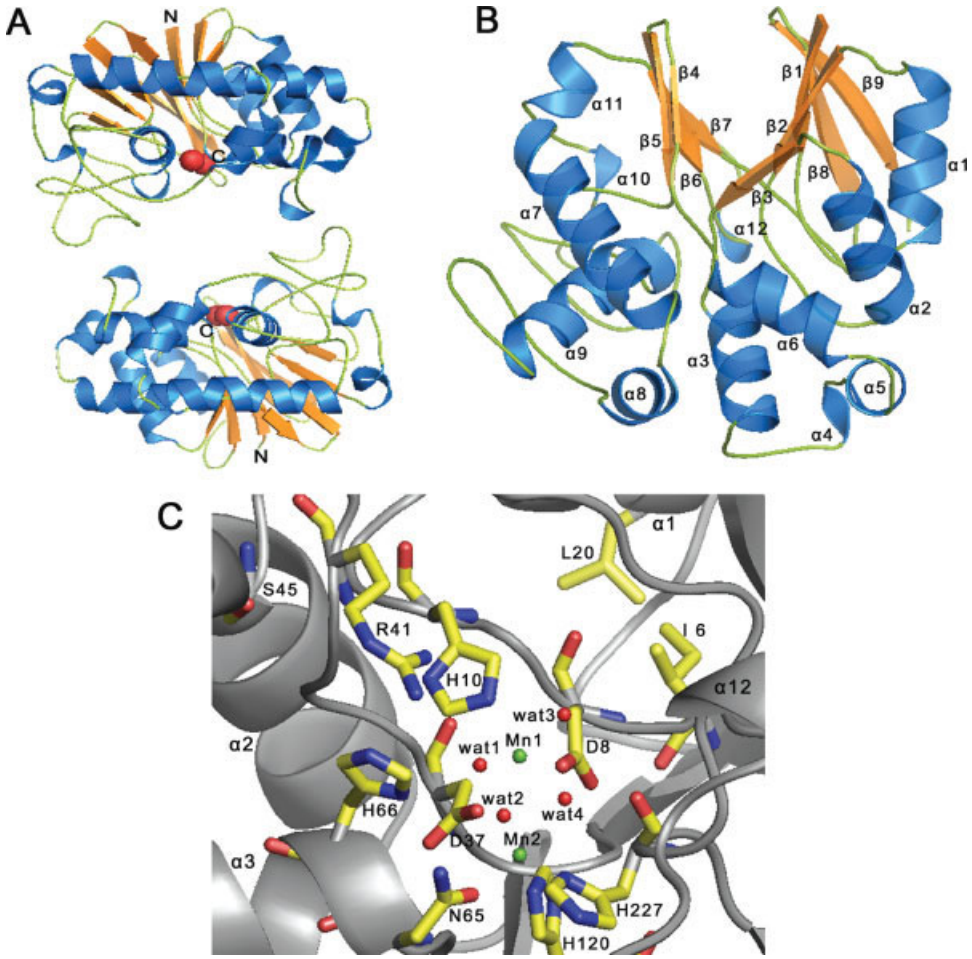

Fig. 1. (**A**) Ribbon representation of the Ap_4A hydrolase dimer in asymmetric unit. (**B**) Ribbon representation of the Ap_4A hydrolase monomer. The α-helix and β-strand are colored marine and orange, respectively. (**C**) Close-up view of the groove with conserved residues, which consists of α1, α2, α3, and α12. The manganese ion (green), water molecule (red), and nearby completely conserved residues are shown. The figure was generated by Pymol (De Lano Scientific).

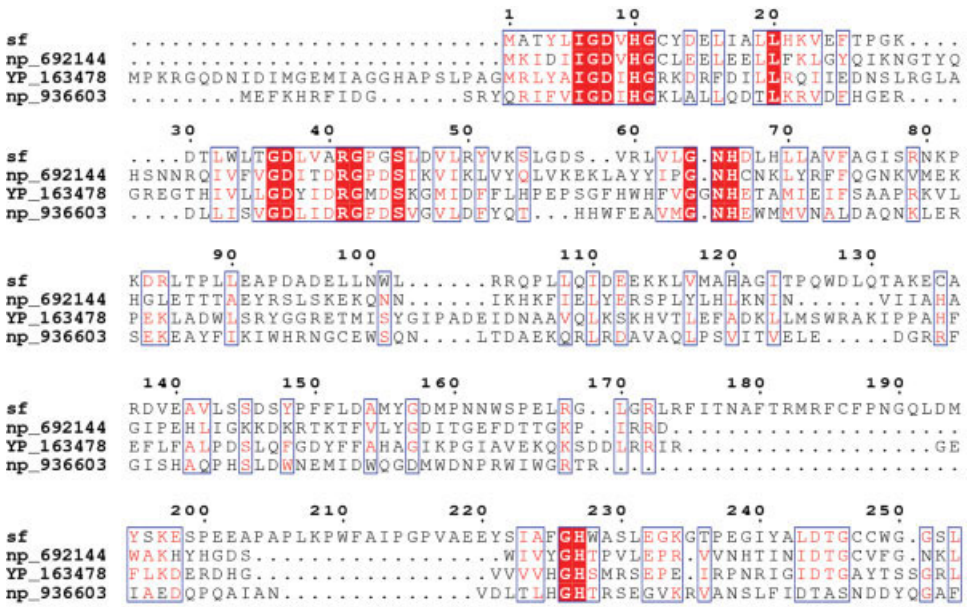

Fig. 2. Sequence comparison between symmetrical Ap_4A hydrolases constructed by CLUSTAL W.[12] sf, *Shigella flexneri 2a;* np_692144, *Buchnera aphidicola*; YP_163478, *Yersinia pestis*; np_936603, *Vibrio vulnificus*. Strictly conserved and similar residues are represented within a red box and by a red letter, respectively. The figure was generated with WebESPript.[13]

REFERENCES

1. Zamecnik PC, Stephenson ML, Janeway CM, Randerath K. Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. Biochem Biophys Res Commun 1966;24:91–97.
2. Goerlich O, Foeckler R, Holler E. Mechanism of synthesis of adenosine(5′) tetraphospho (5′)adenosine (AppppA) by aminoacyl-tRNA synthetases. Eur J Biochem 1982;126:135–142.
3. Kisselev LL, Justesen J, Wolfson AD, Frolova LY. Diadenosine oligophosphates (ApnA), a novel class of signaling molecules? FEBS Lett 1998;427:157–163.
4. Guranowski A. Specific and nonspecific enzyme involved in the catabolism of mononucleoside and dinuceoside polyphosphases. Pharmacol Ther 2000;87:117–139.
5. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. In: Carter CW, Sweet RM, editors. Methods in enzymology, Vol. 276: Macromolecular crystallography, part A. San Diego CA: Academic Press; 1997. pp 307–326.
6. Hendrickson WA. Determination of macromolecular structures from anomalous diffraction of synchrotron radiation. Science 1991;254:51–58.
7. Terwilliger TC, Berendzen J. Automated MAD and MIR structure solution. Acta Crystallogr D Biol Crystallogr 1999;276:307–326.
8. Terwilliger TC. Maximum-likelihood density modification. Acta Crystallogr D Biol Crystallogr 2000;56:965–972.
9. Jones TA, Zou JY, Cowan SW, Kjeldgaard. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr A 1991;47:110–119.
10. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. Acta Cryst D Biol Crystallogr 1997;53:240–255.
11. Collaborative Computational Project 4. The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 1994;50:760–763.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–4680.
13. Gouet P, Courcelle E, Stuart DI, Metoz F. ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics 1999;15:305–308.
14. Scott B, Sedelnikova SE, Blackburn GM, Abdelghany HM, Baker PJ, McLennan AG, Rafferty JB. The crystal structure of diadenosine tetraphosphate hydrolase from *Caenorhabditis elegans* in free and binary complex forms. Structure 2002;10:589,600.
15. Holm L, Sander C. Dali/FSSP classification of three-dimensional protein folds. Nucleic Acids Res 1997;25:231–234.
16. Barton GJ, Cohen PT, Barford D. Conservation analysis and structure prediction of the protein serine/threonine phosphatases. Sequence similarity with diadenosine tetraphosphatase from *Escherichia coli* suggests homology to the protein phosphatases. Eur J Biochem 1994;220:225–237.
17. Voegtli WC, White DJ, Reiter NJ, Rusnak F, Rosenzweig AC. Structure of the bacteriophage λ Ser/Thr protein phosphatase with sulfate ion bound in two coordination modes. Biochemistry 2000;39:15365–15374.