Crystallization and preliminary crystallographic studies of PotA, a membrane-associated ATPase of the spermidine-preferential uptake system in *Thermotoga maritima*

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Received 24 February 2014
 Accepted 15 April 2014

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A membrane-associated ATPase, PotA, is a component of the spermidine-preferential uptake system in prokaryotes that plays an important role in normal cell growth by regulating the cellular polyamine concentration. No three-dimensional structures of membrane-associated ATPases in polyamine-uptake systems have been determined to date. Here, the crystallization and preliminary X-ray diffraction analysis of PotA from *Thermotoga maritima* are reported. Diffraction data were collected and processed to 2.7 Å resolution from both native and selenomethionine-labelled crystals. Preliminary crystallographic analysis revealed that the crystals belonged to the hexagonal space group $P3_112$ (or $P3_212$), with unit-cell parameters $a = b = 88.9$, $c = 221.2$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$, indicating that a dimer was present in the asymmetric unit.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are necessary for cell growth and for the proliferation of all cells (Cohen, 1998; Igarashi & Kashiwagi, 2010). These ubiquitous molecules play important roles in numerous physiological functions such as cell growth and proliferation, nucleic acid and protein synthesis, cell adhesion and repair of the extracellular matrix, and immunity (Casero *et al.*, 1991; Pegg, 1986, 1998; Moinard *et al.*, 2005). Three different polyamine-transport genes in *Escherichia coli* have been cloned and characterized (Igarashi & Kashiwagi, 1999). Two of these constitute the spermidine-preferential (PotABCD) and putrescine-specific (PotFGHI) uptake systems. These polyamine-transport systems, which belong to the ATP-binding cassette (ABC) transporters, consist of a periplasmic substrate-binding protein (PotD or PotF), two transmembrane proteins (PotB and PotC or PotH and PotI) and a membrane-associated ATPase (PotA or PotG). The third transport system, which is catalyzed by PotE, mediates both the uptake and the excretion of putrescine (Kashiwagi *et al.*, 1992, 1997, 2000). The crystal structures of the two substrate-binding proteins (PotD and PotF) complexed with polyamine have been determined previously (Sugiyama, Matsushima *et al.*, 1996; Sugiyama, Vassalyev *et al.*, 1996; Vassalyev *et al.*, 1998). Each of them is divided into two domains with an alternating β - α - β topology, similar to other periplasmic binding proteins. The polyamine-binding site resides in a cleft between two domains as determined by crystallography and site-directed mutagenesis (Kashiwagi *et al.*, 1996; Sugiyama, Matsuo *et al.*, 1996).

In a previous study, we found that the NH₂-terminal domain of PotA is involved in the recognition of ATP and in interactions with a second PotA subunit and with PotB or PotC. The COOH-terminal domain of PotA was also found to be involved in the regulation of ATPase activity and to influence the interactions between PotA and PotB (or PotC) (Kashiwagi *et al.*, 1995, 2002). The polyamines are unique substrates for the ABC transporter, although the specific interactions with the membrane-associated ATPases have never been studied in terms of three-dimensional structure. Therefore, a crystallographic study of PotA at atomic resolution was performed in order to elucidate the role of PotA in a polyamine-uptake system in detail.

Table 1

PotA production information.

Source organism	<i>T. maritima</i>
DNA source	pET-29b-TmPotA expression vector with C-terminal 6×His tag
Forward primer	5'-AGAGCATATGATCGGAGGAGAAGTTTCAAT-3'
Reverse primer	5'-GTGCTCGAGGCCTTCTTCAAGCACCTCTACAATGAA-3'
Expression vector	pET-29b (EMD Biosciences)
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced†	MIGGEVSIKNSKFFDDFQVLKNSLDIKKGEFFSILGPSGC-GKTTLLRVIAAGFEGVESGDVLLDGKSLNLPNKRPNVNIIFQNYALFPHLTVFENIAFPLKLLKLSENEINQRVNELLSLIRMEHAQKMPQLSGGQKQVATARALANEPVLLDEPLSALDAKLRLQELLVELDNLHDRVGITFIYVTHDQAEAISVSDRVALMNEGEIVQVGTPEYEVESPVNVFAATFIGETNLMKAEEVVEDEYVYVESPGIGQFRCYRDEAKKGDRLITLRPEKIRISRKQFRSEETFNVFHGVVDEEYMGHQTKYFVR- LDEGYIMKVYQHARYILDEPIIKWEDEVITWNPDDSFIVEVLEEHHHHHH

† The His tag was retained for the crystallization experiments.

Table 2

Crystallization conditions.

Method	Sitting-drop and hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	6
Buffer composition of protein solution	20 mM potassium phosphate pH 7.5, 300 mM NaCl, 6 mM β-mercaptoethanol, 10% (v/v) glycerol
Composition of reservoir solution	0.1 M HEPES-NaOH pH 8.7, 1.25 M sodium citrate, 2 mM spermine
Volume and ratio of drop	2 µl, 1:1 protein solution:reservoir solution
Volume of reservoir (µl)	1000

2. Materials and methods

2.1. Macromolecule preparation

Thermotoga maritima PotA protein (UniProt entry Q9X196) was prepared as follows. Genomic DNA of *T. maritima* was kindly supplied by Dr K. O. Stetter, Universität Regensburg. To prepare COOH-terminally histidine-tagged PotA protein, DNA fragments encoding PotA were amplified by polymerase chain reaction (PCR) using the primer pair 5'-AGAGCATATGATCGGAGGAGAAGTTTCAAT-3' and 5'-GTGCTCGAGGCCTTCTTCAAGCACCTCTACAATGAA-3' (Table 1). The DNA fragments obtained by PCR were digested with *Nde*I and *Xho*I and inserted into the same restriction sites of pET-29b (EMD Biosciences; pET-29b-TmPotA). *Escherichia coli* BL21(DE3) cells (EMD Biosciences) carrying pET-29b-TmPotA were grown in 2 l modified Luria-Bertani medium and the histidine-tagged PotA was purified using an Ni-NTA column (Qiagen, California, USA). The cell lysate (30 mg protein) in buffer A (20 mM potassium phosphate pH 7.5, 300 mM NaCl, 6 mM β-mercaptoethanol) containing 5 mM imidazole was applied onto a 5 ml Ni-NTA column, washed with buffer A containing 20 mM imidazole and the histidine-tagged PotA was eluted with buffer A containing 100 mM imidazole. The purified histidine-tagged PotA protein consisting of 374 amino-acid residues showed one major band on SDS-PAGE.

2.2. Crystallization

For crystallization, the PotA protein was dissolved in buffer A with 10% (v/v) glycerol and was then concentrated using a Centricon-10 concentrator. The final concentration of the PotA protein was 6 mg ml⁻¹ and it was stored at 193 K. A preliminary search for crystallization conditions was performed by the sitting-drop and hanging-drop vapour-diffusion methods in 24-well Cryschem plates

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	Native	SeMet derivative
Diffraction source	BL41XU, SPring-8	BL41XU, SPring-8
Wavelength (Å)	1.0	0.9788
Temperature (K)	100	100
Detector	Quantum 315 CCD	Quantum 315 CCD
Crystal-to-detector distance (mm)	350	412
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	120	325
Exposure time per image (s)	0.5	15
Space group	<i>P</i> ₃ 12 (or <i>P</i> ₃ 212)	<i>P</i> ₃ 12 (or <i>P</i> ₃ 212)
<i>a</i> , <i>b</i> , <i>c</i> (Å)	88.9, 88.9, 221.2	89.4, 89.4, 222.5
α , β , γ (°)	90, 90, 120	90, 90, 120
Mosaicity (°)	0.7	0.2
Resolution range (Å)	50–2.70 (2.75–2.70)	50–2.70 (2.75–2.70)
Total No. of reflections	140446	424059
No. of unique reflections	27592	53422
Completeness (%)	98.8 (96.8)	97.8 (94.6)
Multiplicity	5.1 (3.2)	7.9 (7.0)
$\langle I/\sigma(I) \rangle$	8.7 (1.4)	6.7 (2.4)
$R_{\text{merge}}^{\dagger}$ (%)	10.7 (48.8)	11.8 (51.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	40.0	50.7

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observed intensity of reflection *hkl* and $\langle I(hkl) \rangle$ is the average intensity over symmetry-equivalent measurements.

(Hampton Research, USA). Typically, a 1.0 µl droplet containing PotA protein at approximately 6 mg ml⁻¹ was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 1 ml reservoir solution. All crystallizations were carried out at 293 K (Table 2).

2.3. Data collection and processing

Data were collected on beamlines BL38B1, BL41XU and BL44XU of the SPring-8 synchrotron-radiation source, Hyogo, Japan. The crystals were soaked in the reservoir solution as a cryoprotectant and were then mounted in a cryoloop. The crystal was directly flash-cooled in a stream of cold nitrogen gas at 100 K. Diffraction data sets were collected using 1.0° oscillations with a crystal-to-detector distance of 350 mm (native data) or 412 mm (SAD data). The diffraction data were processed and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997). The crystal parameters and data-collection statistics for the native and SAD data sets are summarized in Table 3.

Figure 1

Crystals of the PotA protein. Crystals grew as needles within one week.

Figure 2
Diffraction pattern of a PotA crystal. The crystal was grown under the optimum conditions and then flash-cooled in the reservoir solution as a cryoprotectant. (a) Overall image of one shot. (b) Enlargement of the boxed region in (a) with the background level adjusted for clarity.

3. Results and discussion

Initial crystallization trials were carried out using Crystal Screen and Crystal Screen 2 (Hampton Research, USA). Crystals of PotA were obtained with Crystal Screen condition No. 38 (1.4 M trisodium citrate, 0.1 M Na HEPES pH 7.5) and Crystal Screen 2 condition No. 28 (1.6 M trisodium citrate pH 6.5). Microcrystals were observed as clusters from reagents that contained sodium citrate as a precipitant. The crystals from the cluster exhibited poor diffraction quality and were too small for diffraction experiments. The use of a repeated seeding technique in an attempt to improve the crystals was unsuccessful. Since this technique did not seem to be favourable for growing large high-quality crystals, the crystallization conditions were further refined. Consequently, the crystallization conditions were optimized by changing the sodium citrate concentration and the pH. A search for a suitable precipitant solution for crystallization was performed in the range 1.0–1.5 M sodium citrate using the HEPES–NaOH buffer system with pH values ranging from 6.5 to 9.0 at 293 K. As a result, single crystals were obtained from 0.1 M HEPES–NaOH pH 8.7 containing 1.25 M sodium citrate. They grew to maximum dimensions of approximately $0.01 \times 0.01 \times 0.1$ mm. Furthermore, the size of the crystals was improved by the addition of spermidine. Needle-shaped crystals that were large enough for diffraction experiments appeared within one week. The best crystals were obtained at 293 K using 0.1 M HEPES–NaOH pH 8.7, 1.25 M sodium citrate containing 2 mM spermidine (Table 2), resulting in single crystals of high quality. They reached maximum dimensions of $0.02 \times 0.02 \times 0.5$ mm (Fig. 1).

Diffraction studies were performed at cryogenic temperature (100 K). A crystal was mounted in a cryoloop, transferred *via* the reservoir solution as a cryoprotectant and then rapidly cooled in a cryogenic nitrogen-gas stream. X-ray diffraction data were collected at the SPring-8 synchrotron-radiation source. The single crystals diffracted to 2.7 Å resolution using synchrotron radiation (Fig. 2). An analysis of the symmetry and systematic absences in the recorded

diffraction pattern indicates that the crystals belonged to the hexagonal space group $P3_112$ (or $P3_212$), with unit-cell parameters $a = b = 88.9$, $c = 221.2$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient is calculated to be $2.7 \text{ Å}^3 \text{ Da}^{-1}$, with an estimated solvent content of 55.2%. These values are within the range commonly observed for protein crystals (Matthews, 1968). Selenomethionine-substituted PotA protein was produced using the *E. coli* expression system in order to determine the structure using the SAD method. Crystals of the selenomethionine (SeMet) derivative of PotA were obtained using the same conditions.

14 selenium sites were found in the asymmetric unit of the crystal, and the initial experimental phases were calculated at 3.2 Å resolution using *SHELXD* (Sheldrick, 2008) and *HKL2MAP* (Pape & Schneider, 2004). The phases from *SHELXD* and *HKL2MAP* were improved by density modification using *DM* (Cowtan & Main, 1996). Model building and further refinement with *Coot* (Emsley & Cowtan, 2004) are under way.

The authors are grateful to Seiki Baba, Nobuhiro Mizuno, Nobutaka Shimizu, Masahide Kawamoto, Eiki Yamashita, Yasufumi Umena, Masato Yoshimura and Atsushi Nakagawa for their kind help in data collection at BL38B1, BL41XU and BL44XU of SPring-8. The synchrotron-radiation experiments were performed at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI; Proposals 2010A1537, 2010B1261 and 2012A1370). This work was supported by a Core Research for Evolutional Science and Technology (CREST) grant to YM and by JSPS KAKENHI grant Nos. 25286051 and 25650051 to SS.

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