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Expression, purification, crystallization and preliminary crystallographic analysis of spermidine acetyltransferase from *Escherichia coli*

The spermidine acetyltransferase (SAT) from *Escherichia coli* catalyses the transfer of acetyl groups from acetyl-CoA to spermidine. SAT has been expressed and purified from *E. coli*. SAT was crystallized by the sitting-drop vapour-diffusion method to obtain a more detailed insight into the molecular mechanism. Preliminary X-ray diffraction studies revealed that the crystals diffracted to 2.5 Å resolution and belonged to the cubic space group *P*23, with unit-cell parameters $a = b = c = 148.7$ Å. They contained four molecules per asymmetric unit.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are necessary for normal cell growth (Cohen, 1998; Igarashi & Kashiwagi, 2010). Since polyamines interact with nucleic acids and exist primarily as polyamine–RNA complexes in cells (Watanabe *et al.*, 1991; Miyamoto *et al.*, 1993), their proliferative effects are presumed to be caused by changes in RNA function. It was found that polyamines both enhanced the formation of active 30S ribosomal subunits for stimulating general protein synthesis (Echandi & Algranati, 1975; Igarashi *et al.*, 1979, 1981) and stimulated specific kinds of protein synthesis that are important for cell growth and viability (Igarashi & Kashiwagi, 2010). Thus, it is important to understand the mechanism by which the cellular polyamine concentration is regulated. Polyamine content is regulated by the biosynthesis, degradation, uptake and excretion of polyamines. For instance, two different spermidine-transport genes in bacteria have been cloned and characterized (Kashiwagi *et al.*, 1990; Lu *et al.*, 2002). They constitute the spermidine-preferential (PotABCD or SpuEFGH) uptake systems. It was proposed that PotABCD (SpuEFGH) forms an ABC transporter system for spermidine uptake, in which PotD (SpuE) is the periplasmic spermidine-preferential binding protein, PotA (SpuF) is the ATPase and PotB and PotC (SpuG and SpuH) form the transmembrane channel. Structures of PotD in complex with spermidine and of SpuE in complex with spermidine have been reported (Sugiyama, Matsuo *et al.*, 1996; Sugiyama, Matsushima *et al.*, 1996; Sugiyama, Vassilyev *et al.*, 1996; Wu *et al.*, 2012). These structures and mutational analyses (Kashiwagi *et al.*, 1996) provided important insights into the spermidine-binding properties. However, it is still unclear why the asymmetric spermidine binds to PotD (and also to SpuE) directionally with the diamino propane portion of the spermidine pointing to the inner side of the binding pocket.

As part of a polyamine-degradation pathway in prokaryotes, spermidine acetyltransferase (SAT; molecular mass 22 kDa) is a key enzyme that transfers the acetyl group from acetyl-CoA to either the N-1 or N-8 position of spermidine, thereby reducing the intracellular polyamine level (Fukuchi *et al.*, 1994). Weak similarity was also observed to PotD. The fact that similarities are observed in the amino-acid sequence between residues 87 and 141 suggests that the active centre of SAT might be located in the COOH-terminal portion, assuming that the conserved residues reflect essential active-site residues. Although the physiological role of SAT has been extensively

studied, it remains unclear how the enzyme catalyses the reaction and how the activity of the enzyme is regulated at the molecular level.

Here, we report the expression, purification, crystallization and preliminary crystallographic studies of SAT from *Escherichia coli*. The crystal structure will promote further understanding of the molecular mechanism governing the specific asymmetric spermidine recognition by SAT and the catalytic mechanism of SAT.

2. Materials and methods

2.1. Expression

SAT was expressed in *E. coli*. The polymerase chain reaction (PCR) product encoding the *E. coli* SAT open reading frame (ORF) was digested with *Nde*I and *Sal*I restriction enzymes and then cloned into the corresponding sites of a pT7-7 expression vector (Novagen, USA). The resultant expression vector (pT7 SAT) was used to transform *E. coli* BL21 (DE3) cells. The cells were grown in Luria broth medium with 100 µg ml⁻¹ ampicillin at 310 K. Expression of the *speG* gene encoding SAT was induced by adding 0.1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside when the optical density of the medium reached 0.6 at 600 nm and the culture was continued for a further 3 h.

2.2. Purification

The cells were collected by centrifugation at 5000g for 10 min, resuspended in 10 mM HEPES-KOH pH 7.5 containing 10 mM magnesium acetate, 300 mM KCl, 6 mM β-mercaptoethanol, 10 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM spermidine and 0.1 µg ml⁻¹ DNase I, and disrupted by sonication. After centrifugation at 80 000g for 30 min, 2.0% streptomycin (final concentration) was added to the supernatant and the solution was kept on ice for 30 min. After the solution had been centrifuged at 2000g for 10 min, the supernatant was dialysed overnight against 300 volumes of buffer A (10 mM HEPES-KOH pH 7.5, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 10% glycerol, 10 µM PMSF, 0.02% Brij-35, 1 mM spermidine) containing 50 mM KCl. The precipitate was obtained and was solubilized in buffer A by increasing the KCl concentration to 300 mM. This solubilized precipitate was applied onto a Macro-Prep High Q column (Bio-Rad, USA) previously equilibrated with buffer

A containing 300 mM KCl and washed with four column volumes of the same buffer. The other bound proteins and nucleic acids were eluted with a linear 300–1000 mM KCl gradient at a flow rate of 1 ml min⁻¹. SAT was obtained from the unbound and wash fractions. The fraction containing SAT was applied onto a CoA-Sepharose column (1 × 8 cm) that had been synthesized from CoA and Sepharose 4B using the method of Ryan & McClure (1979). After the column had been washed with buffer A containing 300 mM KCl, SAT was eluted with buffer A containing 30 mM ADP and 300 mM KCl. The eluted SAT was further purified using a HiLoad 26/60 Superdex 75 prep-grade column (GE Healthcare, USA) equilibrated with buffer A containing 300 mM KCl. All protein-purification steps were performed at 277 K. The yield of pure protein was about 20.8 mg per litre of culture.

2.3. Crystallization

For crystallization, the purified sample was concentrated to about 60 mg ml⁻¹ by the ultrafiltration method using an Amicon Ultra-10 concentrator (Millipore, USA). The protein concentration was determined using Bio-Rad Protein Assay Kit reagents with bovine serum albumin as the standard. The purity was confirmed by SDS-PAGE on 12.5% gels (Fig. 1). All crystallization experiments were performed at 293 K using the sitting-drop vapour-diffusion method. Initial crystallization screening conditions were constructed using several commercially available kits: Crystal Screen, Crystal Screen 2, Crystal Screen Lite and Crystal Screen Cryo (Hampton Research, USA), Wizard I, II and III (Emerald BioStructures, USA) and Stura FootPrint Screens (Molecular Dimension, UK). Typically, a 1 µl droplet consisting of approximately 40 mg ml⁻¹ SAT in 10 mM HEPES-KOH pH 7.5, 50 mM CoA, 50 mM spermidine, 300 mM KCl, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 10% glycerol, 10 µM PMSF, 0.02% Brij-35 was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 500 µl reservoir solution.

3. Results and discussion

A suitable SAT crystal with dimensions of 0.1 × 0.1 × 0.1 mm appeared at 293 K within a few days (Fig. 2). The best crystallization condition for X-ray diffraction measurement was obtained with a reservoir solution consisting of 50 mM sodium cacodylate pH 6.5, 9% (w/v) PEG 8000, 0.1 M calcium acetate. X-ray diffraction experiments were performed under liquid-nitrogen-cooled conditions at 100 K. A crystal was mounted in a nylon loop, soaked rapidly in 50 mM sodium cacodylate pH 6.5, 15% (w/v) PEG 8000, 20% (v/v)

Figure 1
12.5% SDS-PAGE of purified SAT. Lane M, molecular-mass markers (labelled in kDa); lane 1, 2 µg purified SAT. The gel was stained with Coomassie Brilliant Blue.

Figure 2
Crystals of SAT grown by sitting-drop vapour diffusion.

glycerol and then cooled by submerging it in liquid nitrogen. X-ray diffraction data sets were collected for the SAT crystal on beamline BL41XU at the SPring-8 synchrotron-radiation source, Hyogo, Japan using an ADSC Quantum 315 detector (Fig. 3). The wavelength was fixed at 0.97 Å. A total of 180 frames were recorded with an oscillation angle of 1.0°, an exposure time of 1 s per frame and a crystal-to-detector distance of 300 mm. Diffraction data were processed and

Table 1

Data-collection statistics for SAT.

Values in parentheses are for the outermost resolution shell.

X-ray source	BL41XU, SPring-8
Wavelength (Å)	0.97
Space group	<i>P</i> 23
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = <i>c</i> = 148.7
Resolution (Å)	50–2.50 (2.54–2.50)
No. of reflections	336558
No. of unique reflections	38070
Average multiplicity	8.8 (7.8)
$\langle I/\sigma(I) \rangle$	7.4 (3.2)
Completeness (%)	99.9 (100)
$R_{\text{merge}}^{\dagger}$ (%)	9.4 (40.6)
Mosaicity (°)	0.31
Molecules per asymmetric unit	4
V_M (Å ³ Da ^{−1})	3.1
Solvent content (%)	60.5

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, 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.

scaled using the *HKL-2000* program package (Otwinowski & Minor, 1997). Analysis of the symmetry and of the systematic absences in the recorded diffraction pattern indicated that the crystals belonged to the cubic space group *P*23, with unit-cell parameters *a* = *b* = *c* = 148.7 Å. Assuming the presence of four SAT molecules (4 × 22 kDa) in the asymmetric unit, the V_M value is calculated to be 3.1 Å³ Da^{−1}, with an estimated solvent content of 60.5%; these values are within the range commonly observed for protein crystals (Matthews, 1968). This result suggests that SAT may be comprised of four identical subunits in the asymmetric unit, showing a similar tendency to the previous result that the SAT molecule elutes at a molecular mass of about 95 kDa in gel-filtration chromatography (Fukuchi *et al.*, 1994). A total of 336 558 observed reflections were merged into 38 070 unique reflections in the 50.0–2.5 Å resolution range. Table 1 summarizes the data-collection statistics.

An attempt to determine the structure using the molecular-replacement method with the *MOLREP* (Vagin & Teplov, 2010) program as implemented within the *CCP4* package (Winn *et al.*, 2011) was performed using the coordinates of chain A of a homologous protein (53% sequence identity) from *Vibrio cholerae* (PDB entry 3eg7; Center for Structural Genomics of Infectious Diseases, unpublished work) substituted with polyalanine as a search model. The results of molecular replacement suggest that the asymmetric unit of the crystal contains four SAT molecules. Initial rigid-body and restrained refinements with *REFMAC5* (Murshudov *et al.*, 2011) using the *MOLREP* output model gave an R_{work} of 39.7% and an R_{free} of 49.4%. Further structural refinement is currently under way.

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References

Cohen, S. S. (1998). *A Guide to the Polyamines*. Oxford University Press.

Figure 3
Diffraction patterns of the *E. coli* SAT crystal. The crystal was grown under optimum conditions and then flash-cooled using 20% (v/v) glycerol and 15% (w/v) PEG 8000 as a cryoprotectant. The circles from the edge to the centre indicate 2.5 and 3.0 Å resolution, respectively. (a) X-ray diffraction image at rotation angle 0°. (b) X-ray diffraction image at rotation angle 90°.

- Echandi, G. & Algranati, I. D. (1975). *Biochem. Biophys. Res. Commun.* **62**, 313–319.
- Fukuchi, J., Kashiwagi, K., Takio, K. & Igarashi, K. (1994). *J. Biol. Chem.* **269**, 22581–22585.
- Igarashi, K. & Kashiwagi, K. (2010). *Int. J. Biochem. Cell Biol.* **42**, 39–51.
- Igarashi, K., Kashiwagi, K., Kishida, K., Kakegawa, T. & Hirose, S. (1981). *Eur. J. Biochem.* **114**, 127–131.
- Igarashi, K., Kashiwagi, K., Kishida, K., Watanabe, Y., Kogo, A. & Hirose, S. (1979). *Eur. J. Biochem.* **93**, 345–353.
- Kashiwagi, K., Hosokawa, N., Furuchi, T., Kobayashi, H., Sasakawa, C., Yoshikawa, M. & Igarashi, K. (1990). *J. Biol. Chem.* **265**, 20893–20897.
- Kashiwagi, K., Pistocchi, R., Shibuya, S., Sugiyama, S., Morikawa, K. & Igarashi, K. (1996). *J. Biol. Chem.* **271**, 12205–12208.
- Lu, C.-D., Itoh, Y., Nakada, Y. & Jiang, Y. (2002). *J. Bacteriol.* **184**, 3765–3773.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Miyamoto, S., Kashiwagi, K., Ito, K., Watanabe, S. & Igarashi, K. (1993). *Arch. Biochem. Biophys.* **300**, 63–68.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Ryan, R. L. & McClure, W. O. (1979). *Biochemistry*, **18**, 5357–5365.
- Sugiyama, S., Matsuo, Y., Maenaka, K., Vassilyev, D. G., Matsushima, M., Kashiwagi, K., Igarashi, K. & Morikawa, K. (1996). *Protein Sci.* **5**, 1984–1990.
- Sugiyama, S., Matsushima, M., Saisho, T., Kashiwagi, K., Igarashi, K. & Morikawa, K. (1996). *Acta Cryst.* **D52**, 416–418.
- Sugiyama, S., Vassilyev, D. G., Matsushima, M., Kashiwagi, K., Igarashi, K. & Morikawa, K. (1996). *J. Biol. Chem.* **271**, 9519–9525.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Watanabe, S., Kusama-Eguchi, K., Kobayashi, H. & Igarashi, K. (1991). *J. Biol. Chem.* **266**, 20803–20809.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Wu, D., Lim, S. C., Dong, Y., Wu, J., Tao, F., Zhou, L., Zhang, L.-H. & Song, H. (2012). *J. Mol. Biol.* **416**, 697–712.