Crystallization and preliminary X-ray diffraction analysis of the interaction of *Aeromonas hydrophila* MtaN-1 with S-adenosylhomocysteine

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Prokaryotic 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MtaN) is a multifunctional enzyme that can hydrolyze S-adenosyl-L-homocysteine (SAH) and S-methyl-5'-thioadenosine (MTA) to give S-ribosyl-L-homocysteine (SRH) and S-methyl-5'-thioribose (MTR), respectively. This reaction plays a key role in several metabolic pathways, including biological methylation, polyamine biosynthesis, methionine recycling and bacterial quorum sensing. Structurally, MtaN belongs to the MtnN subfamily of the purine nucleoside phosphorylase (PNP)/uridine phosphorylase (UDP) phosphorylase family. *Aeromonas hydrophila* has two MtnN subfamily proteins: MtaN-1, a periplasmic protein with an N-terminal signal sequence, and MtaN-2, a cytosolic protein. In this study, MtaN-1 from *Aeromonas hydrophila* was successfully expressed and purified using Ni-NTA affinity, Q anion-exchange and gel-filtration chromatography. Crystals of the protein in complex with the substrate SAH were obtained and diffracted to a resolution of 1.4 Å. The crystals belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 102.7$, $c = 118.8$ Å. The asymmetric unit contained two molecules of MtaN-1 complexed with SAH.

1. Introduction

S-Adenosylmethionine (SAM) is an important nucleoside that serves as an activated-group donor in diverse metabolic and biosynthetic reactions mediated by radical SAM enzymes (Parveen & Cornell, 2011). Radical SAM enzymes reductively cleave SAM to generate the highly reactive 5'-deoxyadenosyl radical and catalyze the ensuing reactions by utilizing this radical. During this process, highly reactive product inhibitors of these reactions, such as 5'-methylthioadenosine (MTA), S-adenosylhomocysteine (SAH) and 5'-deoxyadenosine (5'-DOA), can accumulate, resulting in enzyme inhibition (Choi-Rhee & Cronan, 2005; Frey & Magnusson, 2003).

In bacteria, the MtnN subfamily protein MTA/SAH nucleosidase (MtaN) is known as a major source of metabolism of the SAH, MTA and 5'-DOA generated by various SAM-utilization pathways (Parveen & Cornell, 2011; Longshaw *et al.*, 2010). This enzyme can irreversibly hydrolyze the glycosidic bond of MTA, SAH and 5'-DOA to give S-methyl-5'-thioribose (MTR), S-ribosyl-L-homocysteine (SRH) and 5'-deoxyribose, respectively (Duerre, 1962). Thus, inhibition of MtaN activity could cause an accumulation of MTA and SAH

within bacterial cells and thus also inhibit radical SAM enzymes, such as methylases and polyamine synthases, which are essential for bacterial growth (Beeston & Surette, 2002). Various antibacterial drugs that target MtaN have been developed to date, although they appear to have limited efficacy (Longshaw *et al.*, 2010). Thus, an understanding of the underlying mechanisms of MtaN is necessary for the development of new and effective antibacterial treatments.

The Gram-negative, rod-shaped bacterium *Aeromonas hydrophila* is an opportunistic pathogen that infects humans, fish and other lower vertebrates and is found in stagnant and flowing fresh water at the interface of sea water and fresh water (Park *et al.*, 2011). This pathogen greatly affects both human health and animal breeding. Previous studies have found that *A. hydrophila* can cause the following four categories of infection: cellulitis, acute diarrhoeal disease, sepsis and other infections (Davis *et al.*, 1978; Janda & Abbott, 2010). *A. hydrophila* has two MtnN subfamily proteins: MtaN-1 and MtaN-2. MtaN-1 appears to be a periplasmic protein because of the presence of a predicted N-terminal signal sequence for secretion, whereas MtaN-2 is a cytosolic protein owing to the lack of a predicted signal sequence. The presence of periplasmic MtaN-1 is interesting because MtaN should function in the cytosol to metabolize radical products.

A total of 11 bacterial and plant MtaN structures have been solved (Lee *et al.*, 2001, 2003, 2005; Siu *et al.*, 2008) using X-ray crystallography and are available in the Protein Data Bank. However, the crystal structure, molecular function and mechanism of action of the MtaN proteins from *A. hydrophila* remain largely uncharacterized. Moreover, no previous studies have investigated the structure and function of periplasmic MtaN. We reasoned that establishing the three-dimensional structure of MtaN-1 from *A. hydrophila* could possibly provide an understanding of the function of the *A. hydrophila* periplasmic MtaN. Here, we report the purification, crystallization and preliminary X-ray analysis of the intact form of MtaN-1 from *A. hydrophila*.

2. Materials and methods

2.1. Construction of an MtaN-1 expression plasmid

To construct a plasmid expressing *A. hydrophila* MtaN-1 (residues 31–278, lacking the signal sequence), we amplified DNA fragments encoding *A. hydrophila* MtaN-1 (residues 31–278) from the genomic DNA of an *A. hydrophila* strain by PCR using standard methods. The PCR primers were constructed in such a way that cloning of the PCR product into the EcoRI and HindIII sites of the pPROEX-HTA vector (Invitrogen, USA) would generate a protein containing a hexahistidine tag (MSYYHHHHHH), a spacer region (DYDIPTT) and a TEV protease cleavage site (ENLYFQ) at the N-terminus. The primers were MtaN-1_his-6_forward, 5'-CCGGAATTCATGGCGGCCAAGCCG-3', and MtaN-1_his-6_reverse, 5'-CCCAAGCTTTCAGGCTTGTGAAGCCGT-3', and the resulting plasmid was named pPRO-TEXHTA-MtaN-1.

2.2. Expression and purification of the recombinant MtaN-1 protein

Escherichia coli BL21 (DE3) cells were transformed with the pPROTEXHTA-MtaN-1 construct for overexpression. To produce a sufficient amount of protein for structural studies, cells were grown in 1 l Luria–Bertani (LB) broth with 50 µg ml⁻¹ ampicillin at 310 K until an OD₆₀₀ of 0.7–0.9 was reached. Protein overexpression was then induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside to the culture at 303 K. The cells were harvested by centrifugation 7 h after induction and were stored at 193 K until use.

The harvested cell pellets were defrosted, suspended in lysis buffer consisting of 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol and disrupted by sonication. The cell debris was removed by centrifugation at 45 000g for 30 min at 273 K. The resulting supernatant fraction was mixed with Ni-NTA affinity resin (GE Healthcare, USA) that had been pre-incubated with Tris buffer and the mixture was mixed for 30 min at 4°C. The resin was washed with lysis buffer supplemented with 20 mM imidazole. The target protein with the hexahistidine tag was eluted with 30 ml of lysis buffer including 250 mM imidazole. The eluted fractions were analyzed by SDS–PAGE. The fractions containing MtaN-1 were pooled and β-mercaptoethanol was added to a final concentration of 10 mM. This solution was incubated with recombinant TEV protease overnight at 298 K to remove the hexahistidine tag. The reaction mixture was diluted fourfold with 20 mM Tris pH 8.0 buffer and then loaded onto a Q anion-exchange column (HiTrap Q; GE Healthcare, USA) for further purification. The protein was eluted from the column by a salt gradient to 1 M NaCl in 20 mM Tris pH 8.0. The collected fractions containing the MtaN-1 protein were pooled, concentrated using Centriprep concentrators (Millipore, USA) and separated on a HiLoad Superdex 200 gel-filtration column (GE Healthcare, USA) that had been pre-equilibrated with lysis buffer. The purity of the final protein was confirmed by 15% SDS–PAGE and Coomassie Blue staining. The purified protein was stored at 277 K for use within a week or stored frozen at 193 K until use.

2.3. Crystallization and crystallographic data collection

Prior to crystallization, the pooled sample of purified MtaN-1 was concentrated to 10 mg ml⁻¹ using a Vivaspin centrifugal concentrator fitted with a 10 kDa molecular-weight cutoff filter (Millipore, USA). The crystallization conditions were initially screened using Crystal Screen HT, a high-throughput sparse-matrix screening kit (Hampton Research, USA). Crystals were grown by the sitting-drop microbatch method at various temperatures (279, 288 and 295 K). Crystals of the recombinant MtaN-1 protein were obtained by vapour diffusion only at 288 K. To obtain a crystal of MtaN-1 in complex with SAH, the crystal of MtaN-1 was gently removed from the crystallization drop and briefly incubated in a solution consisting of 6%(w/v) polyethylene glycol 4000, 0.1 M sodium acetate trihydrate pH 4.6, 0.5 mM SAH.

For data collection under cryogenic conditions, crystals were removed from the drop, soaked for 3–5 s in a 10 μ l droplet of cryoprotectant solution prepared by adding 25%(v/v) glycerol to the optimized crystallization conditions and then flash-cooled in a liquid-nitrogen stream. For the crystal of MtaN-1 complexed with SAH, we collected X-ray diffraction data at 100 K at a wavelength of 1.0000 Å using an ADSC Q310 CCD detector on beamline 5C of Pohang Light Source (PLS), Republic of Korea. A total of 180 diffraction images were recorded from a single crystal. The complete diffraction data sets were subsequently processed, merged and

Table 1

Diffraction statistics for MtaN-1–SAH.

X-ray source	Beamline 5C, PLS
Crystal system	Trigonal
Space group	$P3_121$ or $P3_221$
Wavelength (Å)	1.000
Unit-cell parameters (Å, °)	$a = b = 102.64$, $c = 118.74$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution (Å)	49.5–1.4
Completeness (%)	99.8 (99.3)
$R_{\text{merge}}^{\dagger}$ (%)	6.4 (33)
Multiplicity	8.8 (6.4)
Average $I/\sigma(I)$	40.5 (4.0)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

scaled with *HKL-2000* (Otwinowski & Minor, 1997). The data-collection statistics are given in Table 1.

3. Results and discussion

Recombinant MtaN-1 was overexpressed and purified with a yield of 12 mg from 1 l LB medium culture using the protein-purification methods described in §2. The purity of the protein sample was >96% as judged by Coomassie-stained SDS–PAGE. The protein sample was successfully crystallized under several conditions from the initial crystallization screening trials by the sitting-drop vapour-diffusion method. Subsequent refinement of the initial conditions resulted in the production of larger-sized crystals of $0.07 \times 0.07 \times 0.03$ mm (Fig. 1) in 2 d using droplets consisting of 1 μ l protein solution (10 mg ml $^{-1}$ protein in 20 mM Tris buffer pH 8.0 containing 150 mM NaCl)

Figure 1

Crystals of *A. hydrophila* MtaN-1 complexed with SAH. The approximate dimensions of the crystals are $0.07 \times 0.07 \times 0.03$ mm.

Figure 2

Representative X-ray diffraction pattern for recombinant *A. hydrophila* MtaN-1 complexed with SAH. The resolution at the edge of the detector is 1.4 Å.

Figure 3

Electron-density map of recombinant *A. hydrophila* MtaN-1 complexed with SAH. The $2F_o - F_c$ electron-density map contoured at 1.0σ is shown in light orange. This figure was generated using PyMOL (DeLano, 2004)

and 1 μ l reservoir solution consisting of 6% (w/v) polyethylene glycol 4000, 0.1 M sodium acetate trihydrate pH 4.6.

We collected diffraction data to 1.4 Å resolution for the complex of MtaN-1 with SAH (Fig. 2). Based on the diffraction data, the crystal belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 102.6$, $c = 118.7$ Å. The asymmetric unit contains two molecules of MtaN-1 complexed with SAH. Analysis of the diffraction along the h , k and l axes clearly demonstrates that the c axis of the crystal is a 3_1 or 3_2 axis. The diffraction data set had a resolution range of 49.5–1.4 Å with 100% completeness and an R_{merge} of 6.4%. Data-collection statistics are given in Table 1. We attempted to determine the structure of *A. hydrophila* MtaN-1 complexed with SAH by the molecular-replacement method with MOLREP (Vagin & Teplyakov, 2010) by utilizing the structure of *Helicobacter pylori* MtaN (9.4% sequence identity; PDB entry 3nm4; Ronning *et al.*, 2010) as a search model. This approach produced a clear solution. Cross-rotation and translation-function calculations confirmed that

two molecules are present in the asymmetric unit; the corresponding Matthews coefficient (Matthews, 1968) and solvent content are $3.25 \text{ Å}^3 \text{ Da}^{-1}$ and 62.2%, respectively. The initial model of MtaN-1 built was refined automatically with PHENIX (Adams *et al.*, 2010; Fig. 3). The results showed that the crystals obtained were a complex of MtaN-1 and SAH. Currently, we are in the process of building a model and refining the structure of the MtaN-1 complex with SAH together with its interpretation.

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