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Expression, crystallization and preliminary X-ray crystallographic analysis of cystathionine γ-synthase (XometB) from *Xanthomonas oryzae* pv. *oryzae*

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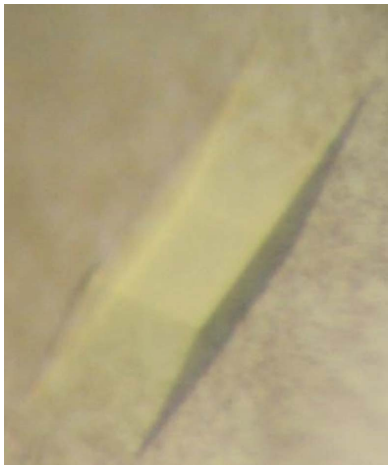

Cystathionine γ-synthase (CGS) catalyzes the first step in the transsulfuration pathway leading to the formation of cystathionine from *O*-succinylhomoserine and L-cysteine through a γ-replacement reaction. As an antibacterial drug target against *Xanthomonas oryzae* pv. *oryzae* (Xoo), CGS from Xoo (XometB) was cloned, expressed, purified and crystallized. The XometB crystal diffracted to 2.4 Å resolution and belonged to the tetragonal space group $I4_1$, with unit-cell parameters $a = b = 165.4$, $c = 241.7$ Å. There were four protomers in the asymmetric unit, with a corresponding solvent content of 73.9%.

1. Introduction

Pyridoxal 5′-phosphate (PLP) dependent enzymes are versatile biocatalysts that can take part in a vast array of reactions such as transamination, racemization, decarboxylation and side-chain elimination and replacement in amino-acid metabolism (Clausen *et al.*, 1996). For decades, PLP-dependent enzymes have been extensively studied, not only because of their functional diversity but also because of their central roles in cysteine, homocysteine and methionine metabolism, which is essential for bacterial growth (Messerschmidt *et al.*, 2003). One example of a PLP-dependent enzyme is cystathionine γ-synthase (CGS).

CGS (EC 4.2.99.9) catalyzes the first step in the transsulfuration pathway leading to the formation of cystathionine from *O*-succinylhomoserine and L-cysteine through a γ-replacement reaction. In the absence of L-cysteine, CGS can perform a γ-elimination reaction to produce α-ketobutyrate, succinate and ammonia (Kaplan & Flavin, 1966; Flavin & Slaughter, 1967; Guggenheim & Flavin, 1968). Moreover, CGS can catalyze a β-elimination reaction as well as a β-replacement and an α- or β-proton-exchange reaction with various substrates (Guggenheim & Flavin, 1969*a*,*b*; Posner & Flavin, 1972). In addition, CGS is regarded as an important drug target for the development of antibacterials against *Escherichia coli*, *Mycobacterium ulcerans* and *Helicobacter pylori*. Structures of CGS from these organisms have been determined at atomic resolution (Clausen *et al.*, 1998; Clifton *et al.*, 2011; Kong *et al.*, 2008).

In *Xanthomonas oryzae* pv. *oryzae* (Xoo), a causal agent of bacterial blight (BB) of rice (*Oryza sativa* L.), CGS is encoded by the *metB* gene (XometB). Even though the whole genome sequence of *X. oryzae* pv. *oryzae* was determined in 2005 (Ochiai *et al.*, 2005; Lee *et al.*, 2005), information on PLP-dependent enzymes in Xoo is still lacking. This study describes the cloning, expression, purification, crystallization and preliminary X-ray crystallographic studies of XometB. A three-dimensional structural study of XometB will elucidate the molecular basis for the enzymatic reaction mechanism of CGS and will be useful for the design of a potential antibacterial drug against Xoo.

2. Materials and methods

2.1. Cloning

The *XometB* gene in *X. oryzae* pv. *oryzae* encodes a total of 405 amino-acid residues. The full-length *XometB* gene was amplified *via* polymerase chain reaction using bacterial cells (Xoo KACC10331

strain) as the template. The sequences of the forward and reverse oligonucleotide primers designed from the published genome sequence (Lee *et al.*, 2005) were as follows: 5′-GGG **CAT ATG** AGC TTT CGT GAC CCC ACC CAC ACC-3′ and 5′-GGG **GGA TCC** TCA CGC GTC GAC TTT TTT ACG GTT-3′, respectively. The bases in bold designate the *Nde*I and *Bam*HI digestion sites. The amplicon was double-digested with *Nde*I and *Bam*HI, ligated into the pET15b expression vector (Novagen) containing a 6×His tag upstream of a thrombin cleavage site, yielding the recombinant clone pET15b-XometB, and transformed into *E. coli* BL21 (DE3) pLysS.

2.2. Overexpression and purification

E. coli BL21 (DE3) pLysS cells containing pET15b-XometB, coding for residues 1–405, were grown at 310 K to an OD_{600} of 0.6 in Luria–Bertani medium supplemented with 100 µg ml^{-1} ampicillin and 37 µg ml^{-1} chloramphenicol. Protein expression was induced by the addition of 0.5 m*M* IPTG. The cells were cultured at 310 K for an additional 4 h. The cells were harvested by centrifugation at 3000*g* (Hanil Supra 30K A1000S-4 rotor, Seoul, Republic of Korea) for 30 min at 277 K. The cell pellet was then resuspended in ice-cold lysis buffer (25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 15 m*M* imidazole, 3 m*M* β-mercaptoethanol) and homogenized by ultrasonication on ice (Sonomasher). The crude cell extract was centrifuged for 1 h at 19 960*g* (Hanil) at 277 K to remove cell debris. The supernatant was applied onto Ni–NTA His-Bind resin (Novagen) and affinity purification was performed according to the manufacturer's protocol at 277 K. The column was washed using lysis buffer including 40 m*M* imidazole. The 6×His-tagged XometB protein was then eluted using lysis buffer containing 250 m*M* imidazole and dialyzed against thrombin cleavage buffer (25 m*M* Tris–HCl pH 7.5, 15 m*M* NaCl, 3 m*M* β-mercaptoethanol) for 16 h at 277 K. After dialysis, the protein was treated with 0.1 U thrombin (Sigma) per milligram of XometB for 6 h at 293 K in order to remove the N-terminal 6×His tag and was again applied onto Ni–NTA His-Bind resin (Novagen) to remove any uncleaved protein. After cleavage, three additional residues (Gly–Ser–His) from the pET15b vector remained at the N-terminus of XometB. The flowthrough fraction containing cleaved XometB protein was collected and applied onto a HiTrap Q ion-exchange column (GE Healthcare) for further purification. The homogeneity of the purified protein was analyzed *via* SDS–PAGE (Fig. 1). For crystallization, the protein solution was concentrated using Centri-Prep (Millipore) to a final concentration of 9 mg ml^{-1} in a buffer consisting of 25 m*M* Tris–HCl pH 7.5, 15 m*M* NaCl, 3 m*M* β-mercaptoethanol.

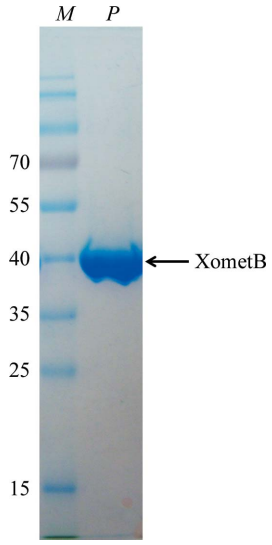

Figure 1
Purified XometB is shown on a 12% SDS–PAGE gel (lane *P*). Lane *M* contains molecular-mass markers (labelled in kDa).

2.3. Crystallization and X-ray data collection

Before setting up the crystallization trials, XometB stock solution (9 mg ml^{-1}) was incubated with 80 µ*M* PLP for 30 min. Initial crystallization was carried out at 287 K by the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Art Robbins) using a Hydra II e-drop automated pipetting system (Matrix) and screening

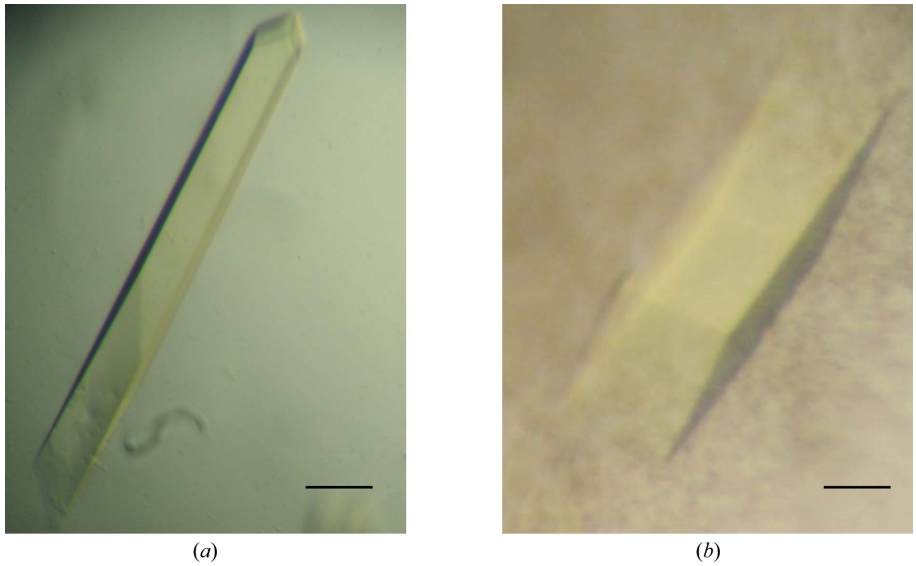

Figure 2
Crystals of XometB. (*a*) Crystal 1: optimized crystals obtained after 3 d using a reservoir solution consisting of 0.2 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5, 14%(*v*/*v*) PEG 400 from Crystal Screen Lite, Hampton Research (0.4 × 0.04 × 0.03 mm). (*b*) Crystal 2: initial crystal of XometB with dimensions of 0.35 × 0.15 × 0.15 mm from condition E7 of the Morpheus screen. This crystal was directly used for X-ray data collection without further optimization. The scale bar represents 0.05 mm

kits from Hampton Research, Emerald BioSystems and Molecular Dimensions (0.5 µl protein solution was mixed with 0.5 µl reservoir solution and equilibrated against 70 µl reservoir solution). After 2 d, small needle-like crystals were observed from several conditions. Crystals were reproduced and optimized using condition No. 23 of Crystal Screen Lite from Hampton Research [0.2 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5, 14%(*v*/*v*) PEG 400] by the hanging-drop method, in which the drops consisted of 0.9 µl protein solution mixed with 0.9 µl reservoir solution. Each hanging drop was positioned over 1 ml reservoir solution. Optimization was achieved by varying the pH and the PEG 400 concentration. Tetragonal needle-shaped crystals with adequate dimensions (crystal 1) were obtained after 3 d using a reservoir solution consisting of 0.2 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5, 14%(*v*/*v*) PEG 400 (Fig. 2*a*). Fully grown crystals (0.4 × 0.04 × 0.03 mm) were flash-cooled at 100 K in liquid nitrogen using 20%(*v*/*v*) PEG 4000, 0.2 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5 as a cryoprotectant. In addition, after 5 d, another well grown crystal (0.35 × 0.15 × 0.15 mm; crystal 2) was observed from the Intelli-Plates using condition E7 of the Morpheus screen (Molecular Dimensions; Gorrec, 2009; Fig. 2*b*). Condition E7 consists of 0.12 *M* Ethylene Glycols Mix (di-ethyleneglycol, tri-ethyleneglycol, tetra-ethyleneglycol and penta-ethyleneglycol), 0.1 *M* Na HEPES/MOPS pH 7.5, 10%(*w*/*v*) PEG 4000, 20%(*v*/*v*) glycerol. This crystal was directly picked up from the drop and flash-cooled at 100 K in liquid nitrogen because this crystallization condition from the Morpheus screen is inherently cryoprotected. X-ray diffraction data were collected from the cryoprotected crystals with 1° oscillation and a crystal-to-detector distance of 300 mm using an ADSC Q315r detector on beamline 5C SBII of the Pohang Light Source (PLS), Republic of Korea. The crystals diffracted to 2.9 Å (crystal 1) and 2.4 Å (crystal 2) resolution, respectively. Diffraction data were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

3. Results and discussion

The XometB crystals were yellow, indicating the presence of an internal Schiff base between Lys and the PLP cofactor. Both crystals belonged to space group $I4_1$. The 2.4 Å resolution data set was used to determine the XometB structure. The unit-cell parameters were $a = b = 165.4$, $c = 241.7$ Å. The space group was derived by auto-indexing (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), there are probably eight molecules in the asymmetric unit, corresponding to a V_M of 2.35 $Å^3$ Da^{-1} and a solvent content of 47.7%. However, molecular replacement (MR) using *Phaser* in the *CCP*4 program package (McCoy *et al.*, 2007) with cystathionine γ-synthase from *E. coli* (PDB entry 1cs1; Clausen *et al.*, 1998; 58.0% sequence identity) as a search model was successful and indicated the presence of four protomers in the asymmetric unit with a high solvent content of 73.9%. The initial *R* factor from the molecular-replacement search was 42.0%; the resulting electron-density maps were of high quality and no clashes were found between molecules. After rigid-body and initial restrained refinement using *REFMAC*5 (Murshudov *et al.*, 2011), the *R* factor decreased to 27.9% and R_{free} was 31.0%. The structure is currently being refined and the final structural details will be described in a separate paper. Our structural data for XometB will provide an insight into its enzymatic mechanism and will be useful for developing a potential antibacterial drug against Xoo.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

	Crystal 1	Crystal 2
X-ray source	Beamline 5C SBII, PLS	Beamline 5C SBII, PLS
Wavelength (Å)	0.97951	0.97951
Unit-cell parameters (Å)	$a = b = 167.2$, $c = 240.3$	$a = b = 165.4$, $c = 241.7$
Space group	$I4_1$	$I4_1$
Resolution	50.0–2.9 (3.0–2.9)	50–2.4 (2.5–2.4)
No. of observations	724456	1170202
No. of unique reflections	160372	122092
Completeness (%)	99.4 (99.8)	100.0 (99.9)
R_{merge}† (%)	17.4 (46.1)	12.8 (46.2)
$\langle I/\sigma(I)\rangle$	13.0 (2.3)	36.5 (5.88)

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

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