Overcoming a hemihedral twinning problem in tetrahydrofolate-dependent *O*-demethylase crystals by the microseeding method

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Received 30 September 2016

Accepted 22 November 2016

Edited by A. Nakagawa, Osaka University, Japan

Keywords: hemihedral twinning; microseeding; cryoprotectant; tetrahydrofolate; *Sphingobium* sp. SYK-6; *O*-demethylase.

Supporting information: this article has supporting information at journals.iucr.org/f

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A tetrahydrofolate-dependent *O*-demethylase, LigM, from *Sphingobium* sp. SYK-6 was crystallized by the hanging-drop vapour-diffusion method. However, the obtained $P3_121$ or $P3_221$ crystals, which diffracted to 2.5–3.3 Å resolution, were hemihedrally twinned. To overcome the twinning problem, microseeding using $P3_121/P3_221$ crystals as microseeds was performed with optimization of the reservoir conditions. As a result, another crystal form was obtained. The newly obtained crystal diffracted to 2.5–3.0 Å resolution and belonged to space group $P2_12_12$, with unit-cell parameters $a = 102.0$, $b = 117.3$, $c = 128.1$ Å. The $P2_12_12$ crystals diffracted to better than 2.0 Å resolution after optimizing the cryoconditions. Phasing using the single anomalous diffraction method was successful at 3.0 Å resolution with a Pt-derivative crystal. This experience suggested that microseeding is an effective method to overcome the twinning problem, even when twinned crystals are utilized as microseeds.

1. Introduction

Hemihedral twinning, which hampers high-quality crystal structure determination, is a known problem in protein crystallography (Parsons, 2003; Dauter, 2003). While detwinning of diffraction data is possible by determining the twin-fraction value (Yeates, 1997), it sometimes degrades the quality of the diffraction data. Furthermore, the crystal quality is not homogeneous throughout the crystal; the value of the twin fraction frequently differs at different positions in a crystal. This type of heterogeneity also hampers the acquisition of high-quality diffraction data. The fundamental resolution of crystal twinning should therefore be achieved by obtaining an alternate crystal lattice. In some cases, a change in the crystal lattice can be achieved by optimizing the expression construct: removing N-terminal or C-terminal residues or residues at both termini, resulting in a change in crystal packing (MacElrevey & Wedekind, 2005; Malawski *et al.*, 2006). However, since optimization of the expression construct is a rather time-consuming process, simpler methods that could be tried first are desirable. Here, we present a simple method for overcoming the crystal twinning of an *O*-demethylase, LigM, from *Sphingobium* sp. SYK-6 (hereafter referred to as SYK-6; Abe *et al.*, 2005; Masai *et al.*, 2007).

SYK-6, which was isolated from pulping waste liquor, can grow on low-molecular-weight (LMW) lignins with two aromatic rings (Katayama *et al.*, 1988), including β -aryl ethers

(Masai *et al.*, 1991, 1993, 2003; Sato *et al.*, 2009), biphenyl (Peng *et al.*, 1998, 1999; Yoshikata *et al.*, 2014) and diarylpropane, as its sole carbon and energy source. In the degradation pathway of LMW lignins, *O*-demethylation reactions catalyzed by LigM and DesA are key reactions to form catecholic derivatives, which are further degraded by extradiol dioxygenases (Masai *et al.*, 2004; Abe *et al.*, 2005; Fig. 1). In SYK-6, vanillate (VNL) and 3-*O*-methylgallate (3MGA) are converted to protocatechuate (PCA) and gallate, respectively, by LigM, and syringate (SYR) is converted to 3MGA by DesA. Interestingly, LigM and DesA transfer a methyl moiety (C1 unit) of the substrate to their cofactor tetrahydrofolate (THF), forming 5-CH₃-THF, which is then converted to 5,10-CH₂-THF by MetF (Masai *et al.*, 2007). 5,10-CH₂-THF is a key metabolite in the C1 metabolic pathway. While most organisms utilize the glycine-cleavage system (GCS) to synthesize 5,10-CH₂-THF, no GCS is found in SYK-6. Since LigM and DesA show weak sequence similarity (approximately 25% sequence identity) to the T-protein (EC 2.1.2.10) in GCS, SYK-6 might have evolved a unique pathway for 5,10-CH₂-THF synthesis compared with the T-protein in GCS. To understand the evolutionary relationship between the T-protein and LigM, we initiated the crystal structure analysis of LigM.

While crystals of LigM were easily obtained by typical crystallization screening, the initial LigM crystals obtained were hemihedrally twinned. To overcome the twinning problem, we utilized the microseeding method (Stura &

Wilson, 1990) and succeeded in obtaining crystals without twinning. We then optimized the cryoconditions, which allowed us to obtain high-resolution diffraction data. Here, we propose that the microseeding method can be utilized to overcome crystal twinning.

2. Materials and methods

2.1. Protein purification

LigM was produced with an intein–chitin-binding domain (intein-CBD) tag. An expression plasmid for LigM (GenBank BAK65949; Abe *et al.*, 2005; Masai *et al.*, 2012) was constructed using the pTXB1 vector (New England Biolabs). *Escherichia coli* BL21(DE3) cells were transformed with the expression plasmid. The recombinant cells were grown in lysogeny broth medium and the expression of LigM was induced by 0.1 mM IPTG at 291 K when the culture reached an OD₆₀₀ of 0.6–0.7. 24 h after induction, the cells were harvested and washed twice with 20 mM Tris–HCl pH 8.5 containing 300 mM NaCl and 10% (v/v) glycerol. The cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin) and then further disrupted by sonication using a VP-30S (TAITEC). Cell debris and larger particles were removed by centrifugation at 20 000g for 30 min at 277 K. The obtained supernatant was used for purification.

The supernatant solution was incubated on a rotary shaker with chitin beads (New England Biolabs), which had

Figure 1
Generation of one-carbon THF by *O*-demethylation of LMW lignins (a) and by the glycine-cleavage system (b).

Table 1

Macromolecule-production information.

Source organism	<i>Sphingobium</i> sp. SYK-6
DNA source	Genomic DNA
Forward primer	CTTTAAGAGGAGATATACATATGTGCGGCACCTA- CCAAT
Reverse primer	CATCTCCCGTGATGCAGGAAGAGCCGCCGTGAC- GGCAGC
Cloning vector	pTXB1
Expression vector	pTXB1-LigM
Expression host	<i>Escherichia coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MSAPTNLEQVLAAGGNTVEMLRNSQIGAYVYPVV- APEFSNWRTEQWAWRNSAVLFDQTHHMDLYI- RGKDALKLLSDTMINSKPGWEPNKAQYVPVT- PYGHVIGDGIIFYLAEFEFVYVGRAPAAWLM- YHAQTGGYNVDIVHDDRSRPMGKPVQRISW- RFQIQGPKAWDVIEKLHGGTLEKLKFFNMAEM- NIAGMKIRTLRHGMAGAPGLEIWGPYETQEKA- RNAILEAGKEFGLIPVGSRAVPSNTLESGWIP- SPLPAIYTGDKLKAYREWLPANSYEASGAIGG- SFVSSNIEDYYVNPYEIGYGPVVKFDHDFGR- DALEAIDPATQRKKVTLAWNGDDMAKIYASLF- DTEADAHYKFFDLPLANYANTNADAVLDAAGN- VVGMSMFTGYSYNEKRALSLATIDHEIPVGTE- LTVLWGEENGGRKTTTVEPHKQMAVRVAVSPV- PYSVTARETYEGGWRKAATAGSS

previously been equilibrated in 20 mM Tris–HCl pH 8.5, 300 mM NaCl, 10% (v/v) glycerol (buffer *A*) at 277 K for 1 h. The mixture was then loaded onto an Econo-Column (Bio-Rad Laboratories) and washed with buffer *A* to reduce the nonspecific binding of proteins, while retaining the tagged proteins bound to the matrix. On-column intein cleavage was induced by adding 20 mM Tris–HCl pH 8.5, 300 mM NaCl, 10% (v/v) glycerol, 50 mM DTT (buffer *B*) and incubating at 293 K for 40 h. LigM was then eluted using buffer *B*. The eluted LigM was further purified by gel-filtration column chromatography using a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with 20 mM HEPES–NaOH pH 8.5, 150 mM NaCl, 10% (v/v) glycerol (buffer *C*). The purified LigM was concentrated to 20 mg ml^{−1} for crystallization experiments using Amicon Ultra and Centricon centrifugal filter units (Merck Millipore). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The initial crystallization screening of LigM was carried out at 293 K by the sitting-drop vapour-diffusion method using a PXS crystallization robot developed in our laboratory (Hiraki *et al.*, 2006). Crystal Screen and Crystal Screen 2 (Hampton Research; Jancarik & Kim, 1991), PEG/Ion and PEG/Ion 2 (Hampton Research), Crystal Screen Cryo and Crystal Screen Cryo 2 (Hampton Research), MembFac (Hampton Research), FootPrint (Molecular Dimensions), Wizard I and Wizard II (Rigaku Reagents), The PEGs II Suite (Qiagen) and The Protein Complex Suite (Qiagen) were used as reservoir solutions. The sitting drop was prepared by mixing 0.2 µl each of the protein and reservoir solutions and was equilibrated against 180 µl reservoir solution. Artificial mother liquor for *P2₁2₁2* crystals was prepared on the basis of the crystallization conditions [100 mM Tris pH 8.0, 100 mM LiCl, 20% (w/v) PEG 8000]. Crystals of the LigM–VNL complex were prepared by

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	VDX plate
Temperature (K)	293
Protein concentration (mg ml ^{−1})	10
Buffer composition of protein solution	20 mM HEPES pH 8.5, 150 mM NaCl, 10% (v/v) glycerol
Composition of reservoir solution	100 mM Tris pH 8.0, 100 mM LiCl, 20% (w/v) PEG 8000
Standard buffer solution	100 mM Tris pH 8.0, 100 mM LiCl, 25% (w/v) PEG 8000
Volume and ratio of drop	1 µl:1 µl:0.2 µl protein:crystallization solution:seed
Volume of reservoir	0.5

soaking native LigM crystals in the artificial mother liquor containing 10 mM VNL.

2.3. Microseeding

A seed-stock solution was prepared using twinned crystals, which were produced in 100 mM Tris pH 7.5–8.5, 400–800 mM LiCl, 30–35% (w/v) PEG 4000. The twinned crystals were needle-shaped crystals with typical dimensions of 0.50 × 0.05 × 0.01 mm. Microseeds were prepared by breaking approximately ten twinned crystals in 950 µl of the corresponding reservoir solution using a VCX-130 ultrasonic processor (output 50%, 6 × 10 s at 20 s intervals; Sonics & Materials Inc.). The crystal solution was in a 1.5 ml vial and a 6 mm probe was used to break the crystals (titanium alloy Ti-6Al-4V). The obtained microseeds were utilized as a seed stock for microseeding. The seed-stock solution was diluted 100-fold and used for microseeding. A droplet for crystallization was prepared by mixing equal volumes (1 µl) of protein solution and reservoir solution and 0.2 µl of the microseed solution. The droplet was then equilibrated with reservoir solution in a hanging-drop setup at 293 K (Table 2). The seed stock was stored at −20°C and could be utilized several times. When the seed stock became ineffective, a new seed stock was prepared.

2.4. Data collection and processing

Diffraction data for the LigM crystal were collected at 95 K using a PILATUS 2M-F detector on beamline NE-3A of the Photon Factory Advanced Ring (PF-AR; KEK, Tsukuba, Japan), using a PILATUS3 S6M detector on beamline BL17A of the Photon Factory (PF) and using a MARmosaic 300 detector on beamline 23-ID-B of the Advanced Photon Source (APS; Argonne National Laboratory, USA). The *P2₁2₁2* crystals were cryoprotected before data collection by soaking them in the artificial mother liquor supplemented with 20% (v/v) ethylene glycol. A heavy-atom derivative crystal was prepared by the soaking method; a LigM crystal was soaked in a standard buffer solution [100 mM Tris pH 8.0, 100 mM LiCl, 25% (w/v) PEG 8000] containing 5 mM ammonium tetrachloroplatinate(II) for 40 min and flash-cooled in liquid nitrogen. The diffraction data for single-wavelength anomalous diffraction (SAD) phasing were collected using X-rays with a wavelength of 1.0716 Å; this wavelength was chosen based on the X-ray absorption spectrum (XAS) for Pt. The

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Protein	LigM, native	LigM-VNL	LigM, derivative	LigM $P3_1(2)21^\dagger$
Diffraction source	BL17A, PF	23-ID-B, APS	NE-3A, PF-AR	BL17A, PF
Wavelength (Å)	0.9800	1.0332	1.0716	0.9800
Temperature (K)	95	95	95	95
Detector	PILATUS3 S6M	MAmosaic 300	PILATUS 2M-F	PILATUS3 S6M
Crystal-to-detector distance (mm)	295	250	313	577
Rotation range per image (°)	0.2	0.5	0.5	0.1
Total rotation range (°)	0–360	10–190	0–360 × 4	0–360
Exposure time per image (s)	0.2	1.0	0.2	0.1
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P3_121$ or $P3_221$
a, b, c (Å)	103.4, 118.7, 127.9	102.7, 117.7, 129.7	102.0, 117.3, 128.1	112.2, 112.2, 218.9
Mosaicity (°)	0.10	0.13	0.19	0.10
Resolution (Å)	49.45–1.90 (1.93–1.90)	47.64–1.90 (1.93–1.90)	49.23–3.00 (3.16–3.00)	45.58–2.70 (2.80–2.70)
Total No. of reflections	1625285	903986	2065452	878885
No. of unique reflections	124319	123835	31457	44178
Completeness (%)	100.0 (100.0)	100.0 (99.9)	100.0 (100.0)	99.4 (98.4)
Multiplicity	13.1 (13.7)	7.3 (5.9)	65.7 (62.9)	19.9 (17.6)
$\langle I/\sigma(I) \rangle$	10.9 (2.2)	14.2 (2.4)	36.2 (12.2)	11.0 (2.4)
R_{meas} (%)	20.9 (133.9)	14.7 (96.4)	20.3 (59.4)	32.3 (142.9)
Overall B factor from Wilson plot (Å ²)	12.5	17.0	26.4	37.4

† Diffraction data from an untwinned $P3_121$ or $P3_221$ crystal.

diffraction data sets were processed and scaled using *XDS* (Kabsch, 2010) and *AIMLESS* (Evans & Murshudov, 2013), respectively. Crystal twinning was analyzed with *POINTLESS* in the *CCP4* program suite (Winn *et al.*, 2011).

3. Results and discussion

LigM was successfully expressed in *E. coli* BL21 (DE3) and purified to near-homogeneity as assessed by SDS–PAGE (Supplementary Fig. S1). The initial crystallization screening of LigM gave thin needle-shaped crystals from Crystal Screen condition No. 18 [100 mM MES pH 6.5, 200 mM magnesium acetate tetrahydrate, 20% (w/v) PEG 8000] and The PEGs II Suite condition No. 83 [100 mM Tris pH 8.5, 500 mM LiCl, 28% (w/v) PEG 6000]. Based on these conditions, the buffer (MES, bis-tris, Tris), the pH (7.0–8.5), the concentration of LiCl (100–1500 mM) and the PEG (PEG 2000 MME, PEG 4000, PEG 6000 and PEG 8000) were systematically optimized by the hanging-drop vapour-diffusion method. As a result of the optimization, larger needle-shaped crystals were obtained from a reservoir solution consisting of 100 mM Tris pH 7.5–8.5, 400–800 mM LiCl, 30–35% (w/v) PEG 4000. The diffraction data collection revealed that the crystals diffracted to 2.5–3.3 Å resolution and belonged to space group $P3_121$ (or $P3_221$), with unit-cell parameters $a = b = 112.5$, $c = 219.5$ Å (Table 3). An *L*-test in *POINTLESS* (Evans, 2011), however, showed that about 50% of the obtained crystals were twinned, with an average twin fraction of 0.21. The *L*-test was developed by the Yeates group as a robust method to detect crystal twinning and is frequently utilized (Padilla & Yeates, 2003). The average resolution of the $P3_121$ or $P3_221$ crystals was approximately 2.9 Å.

Optimization of an expression construct is one possible method of overcoming the crystal twinning, particularly in multi-domain proteins such as eukaryotic signalling proteins.

However, LigM is a bacterial enzyme with limited possibilities for construct optimization. We therefore tried a different strategy. Microseeding has frequently been utilized to increase the reproducibility in crystallization; this method can sometimes yield different crystal forms, probably because it takes advantage of the nucleation of crystals (Shaw Stewart *et al.*, 2011). We therefore utilized the microseeding method with previously obtained $P3_121$ or $P3_221$ crystals.

Microseeding was performed coupled with optimization of the reservoir solution. Based on the crystallization conditions that produced the $P3_121$ or $P3_221$ crystals, we optimized the PEG (PEG 2000 MME, PEG 4000, PEG 6000 and PEG 8000), the concentration of LiCl (100–1000 mM) and the pH of the buffer (7.0–8.5). Most of the crystallization conditions with microseeds gave needle-shaped crystals that belonged to space group $P3_121$ or $P3_221$. However, the crystallization condition with PEG 8000 [100 mM Tris pH 8.0, 100 mM LiCl, 20–25% (w/v) PEG 8000] gave plate-shaped crystals (Fig. 2).

Figure 2
 $P2_12_12$ crystals of LigM. The largest dimension of a crystal is approximately 500 µm.

Preliminary crystallographic analysis demonstrated that the plate-shaped crystals belonged to space group $P2_12_12$, which theoretically cannot exhibit hemihedral twinning. Interestingly, in our experiments, while PEG 8000-containing conditions without microseeds always gave the $P3_121$ or $P3_22$ crystals, the same condition with microseeds always gave $P2_12_12$ crystals. These newly obtained $P2_12_12$ crystals were used in subsequent runs of microseeding to crystallize the $P2_12_12$ crystals. The microseeds were stored and utilized several times.

While the twinning problem was successfully overcome with the newly obtained $P2_12_12$ crystals, these crystals diffracted only to 2.5–3.0 Å resolution using glycerol as a cryoprotectant. Screening of the cryoprotectant was then performed. Three or four cryoprotected crystals were prepared for each cryoprotectant. The maximum resolution of the crystals was determined by the visual inspection of snapshot images. Ethylene glycol, diethylene glycol, PEG 200, PEG 400, polyvinylpyrrolidone, sucrose and sorbitol were tested as cryoprotectants, and ethylene glycol gave the best results; the crystals cryoprotected with ethylene glycol diffracted to better than 2.0 Å resolution (Fig. 3; Table 3). We then collected diffraction data for both the native and VNL-complex forms. Assuming three subunits of LigM (52.3 kDa) in the asymmetric unit of the crystal, the Matthews coefficient (V_M) was calculated to be $2.49 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 50.6% (Matthews, 1968).

Heavy-atom derivative screening revealed that ammonium tetrachloroplatinate(II)-soaked crystals were suitable for diffraction data collection; the derivative crystal typically

diffracted to approximately 3.0 Å resolution. The diffraction data of the Pt-derivative crystal could be processed and scaled at 3.0 Å resolution (Table 3). An anomalous difference Patterson map at 3.0 Å resolution showed some clear peaks in the Harker sections (Supplementary Fig. S2). SAD phasing was then performed using *autoSHARP*, resulting in a clear electron-density map (Vonnrhein *et al.*, 2007; Supplementary Fig. S3). Model building and crystallographic refinement are in progress.

In this study, we have shown that microseeding is an effective way to overcome a hemihedral twinning problem and that twinned crystals can be used as microseeds. Since this is a very simple method, we propose the microseeding method as an effective technique for overcoming twinning problems.

Acknowledgements

This work was supported by Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science (JSPS) and the ‘Course-by-Course Education Program to Develop Student Research Capability and Aptitude’ of SOKENDAI to AH, and in part by JSPS KAKENHI 16K14908 to YS. GM/CA@APS is funded in whole or in part with federal funds from the National Cancer Institute (ACB-12002) and the National Institute of General Medical Sciences (AGM-12006). This research used resources of the Advanced Photon Source, a US Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

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Figure 3
X-ray diffraction pattern of an LigM crystal. The diffraction data were collected on BL17A of PF using a PILATUS3 S6M detector. The dotted circle corresponds to a resolution of 2.0 Å.

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