International Journal of Multidisciplinary Research and Development

IJMRD 2014; 1(4): 19-23
www.allsubjectjournal.com
Received: 29.08.2014
Accepted: 15-09-2014
e-ISSN: 2349-4182
p-ISSN: 2349-5979

Pin-Kuei Fu
[d]Division of Critical Care & Respiratory Therapy, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, 40705, Taiwan, R.O.C
[e] Department of Applied Chemistry, National Chi Nan University, Nantou County, 54561, Taiwan, R.O.C
[¥]These authors contributed equally to this submission.

Shih-Ting Tseng
[b]Department of Food and Nutrition, Providence University
[e]Department of Endocrinology and Metabolism, Kuang Tien
[¥]These authors contributed equally to this submission.

Sheng-Chia Chen, Jai-Shin Liu, Ya-Ping Huang, Chia-Shin Yang, Yeh Chen
Department of Biotechnology, Hungkuang University, Taichung 433, Taiwan.

Cheng-Yang Huang
[f]School of Biomedical Sciences, Chung Shan Medical University, No. 110, Sec. 1, Chien-Kuo N. Rd, Taichung City, Taiwan
[g]Department of Medical Research, Chung Shan Medical University Hospital, No. 110, Sec. 1, Chien-Kuo N. Rd, Taichung City, Taiwan.

Correspondence:
Cheng-Yang Huang
School of Biomedical Sciences, Chung Shan Medical University, No. 110, Sec. 1, Chien-Kuo N. Rd, Taichung City, Taiwan

Yeh Chen
Department of Biotechnology, Hungkuang University, Taichung 433, Taiwan.

Crystallization and preliminary X-ray diffraction analysis of SibL, a SAM-dependent C-methyltransferase from *Streptosporangium sibiricum*

Pin-Kuei Fu, Shih-Ting Tseng, Sheng-Chia Chen, Jai-Shin Liu, Ya-Ping Huang, Chia-Shin Yang, Cheng-Yang Huang and Yeh Chen

Abstract
SibL is an S-adenosyl-L-methionine (SAM)-dependent C-methyltransferase (CMT) involved in sibiromycin biosynthesis. Specifically, it establishes the C8 methylation step in the biosynthesis pathway, and the resultant methyl group is important for maturation of the antitumor antibiotic. Here, we purified and crystallized SibL from *Streptosporangium sibiricum* using the sitting-drop vapor-diffusion method. The crystals diffracted to a resolution of 2.9 Å and belonged to orthorhombic space group *F*222, with unit-cell parameters a = 102.93, b = 295.42, c = 322.23 Å. Determining the structure will provide insights into how SibL methylates 3-hydroxykynurenine (3HK).

Keywords: sibiromycin; methyltransferase; antibiotic; SibL, 3-hydroxykynurenine.

Synopsis: 3-hydroxykynurenine C-methyltransferase SibL was crystallized. Determination of its structure will reveal the residues critical for methylation of 3-hydroxykynurenine.

1. Introduction
Sibiromycin is an antitumor antibiotic produced by a actinomycete, *Streptosporangium sibiricum*, and belongs to the class of pyrrolo [1, 4] benzodiazepines (PBDs) (Gauze *et al*, 1969). The PBDs consist of a tricyclic 6-7-5 system with the significant ability against bacteria and cancer by sequence-specific DNA alkylation (Kumar & Lown, 2003). In this alkylation reaction, a covalent linkage is formed between the C-11 of a PBD and the N-2 of a guanine base upon binding in the minor groove of double-stranded DNA, causing slightly distortion of DNA structure (Kopka *et al*, 1994). This leads to the poor recognition of DNA damage by repair proteins, which results in greater cell toxicity (Clingen *et al*, 2005). Although sibiromycin is one of the most potent antitumor antibiotic, testing of sibiromycin encounters difficulties in clinical trials due to a dose-dependent cardiotoxicity (Hurley & Thurston, 1984). Elimination of C-9 hydroxyl group of sibiromycin can reduce cardiotoxicity while still possess antitumor properties (Yonemoto *et al*, 2012). In addition, sibiromycin is one of only two well-known glycosylated PBDs. The O-glycosylation at C7 position significantly increases DNA-binding affinity and therefore sibiromycin has a higher potency than other PBDs (Itoh *et al*, 1988).
The biosynthesis of sibiromycin occurs via a complex biochemical pathway involving more than 10 enzyme-mediated steps (Giessen *et al*, 2011; Li *et al*, 2009). The biochemical study has shown that the anthranilate moiety of sibiromycin is derived from tryptophan via the kynurenine pathway and 3HK is considered as an intermediate. In the biosynthesis of anthranilate moiety, the functions of SibC, -K and -P in the kynurenine pathway were assigned, mainly based on the sequence similarity to their counterparts, kynurenine 3-monoxygenase, aryl formamidase and tryptophan-2, 3-dioxygenase, respectively (Li *et al*, 2009). Experimental evidence for the functions of SibE, -G, -L and -Q among the catalytic Sib proteins was also recently reported (Giessen *et al*, 2011). Briefly described, SibC, -K and -P involve in the formation of 3HK. The following enzyme SibL methylate 3HK to yield 3-hydroxy-4-methylkynurenine (3H4MK) and then SibQ catalyzes the hydrolysis of 3H4MK to give 3-hydroxy-4-methylanthranilic acid (3H4MAA). Finally, SibD and -E participate in synthesizing the tricyclic portion of sibiromycin aglycone. To date, several studies have shown the functions of Sib proteins, whereas the crystal structures of Sib proteins have been not yet been reported on.

SibL is an SAM-dependent C-methyltransferase that consists of 344 amino acids, and the sequence contains the DVGGG motif that is characteristic for the SAM-binding site (Wolters *et al*, 2013). Several homologous enzymes related to SibL in various *Streptomyces* species have been described to date. For instance, SibL shares sequence homology and functional similarity with AcmI and AcmL. AcmI and AcmL are also SAM-dependent aromatic C-methyltransferases involved in the actinomycin biosynthesis and have ability to methylate 3HK at C4 position (Crnovcic *et al*, 2010). However, substrate preference of SibL is slightly different from AcmI/AcmL. The previous studies have also shown that AcmI and AcmL have tyrosine methyltransferase activity, whereas SibL only uses 3HK as a substrate in vitro (Crnovcic *et al*, 2010; Giessen *et al*, 2011). Therefore, the use of crystallography to determine the structure of SibL was reasoned likely to prove a useful approach for understanding its substrate specificity. Here, we report on the crystallization and preliminary crystallographic analysis of SibL.

2. Materials and Methods

2.1 Cloning, expression, and purification of SibL

SibL was obtained by PCR amplification using *S. sibiricum* genomics DNA as a template and was cloned into a T7 promoter-driven expression system (pET-21b) to allow expression of a His_6-tagged recombinant SibL protein in *Escherichia coli* BL21 (DE3) cells. The bacteria were grown at 310 K and 200 rev min^{-1} in 1 L LB medium containing 50 mg l^{-1} ampicillin as a selection marker to an optical density at 600 nm (OD_{600}) of 0.6. The expression of SibL was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1.0 mM and the culture was grown at 293 K for 16 h. E. coli cells from a 4 L culture were harvested by centrifugation at 5,000 × g for 20 min. *E. coli* cells were harvested by centrifugation, and the cell pellet was resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, and 5 mM imidazole). Subsequently, the cells were disrupted by sonication and the crude lysate was centrifuged at 20000 × g for 90 min at 277 K. The clarified supernatant was applied to Ni-NTA His-bind resin pre-equilibrated with binding buffer. Impurities were removed with Ni-NTA wash buffer (50 mM Tris-HCl, 500 mM NaCl, and 10 mM imidazole, pH 8.0), and the bound SibL was eluted with a 0–200 mM linear gradient of imidazole. Fractions containing SibL were pooled, concentrated by ultrafiltration using an Amicon Ultra-15 3K Centrifugal Filter Device (Millipore; 3-kDa cutoff), and loaded onto a HiLoad 16/60 Superdex-200 size-exclusion column (GE Healthcare) equilibrated in gel filtration buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol, and 2 mM TCEP). The fractions containing SibL were pooled and concentrated to 10 mg ml^{-1} for crystallization screening.

2.2 Crystallization and data collection

Initial crystallization trials were performed with commercially available kits (Hampton Research, Emerald BioStructures, and Molecular Dimensions) by using the sitting-drop vapor-diffusion method in 24-well VDX plates (Hampton Research). 1 µl of a protein solution was mixed with an equal amount of mother liquid. Apo-form SibL crystals were obtained after 1 month using 50 mM HEPES buffer (pH 7.0), 40% (v/v) tacsimate (pH 7.0), 2 mM spermine and 2 mM hexamine cobalt (III) chloride. Crystals of SibL were successfully cooled in liquid nitrogen by flash cooling them in the mother liquor supplemented within 20% glycerol as a cryoprotectant for approximately 15 s. X-ray diffraction data were collected at the National Synchrotron Radiation Research Center (NSRRC) BL15A1 in Taiwan. All diffraction images were indexed and integrated using HKL2000 processing software (Otwinowski & Minor, 1997). Details with regards to the statistics for the diffraction data are given in Table I.

Table 1: X-Ray Data Collection Statistics.

Data collection	SibL
Wavelength (Å)	0.9794
Space group	*F*222
Unit cell (Å)	*a* = 102.93
	b = 295.42
	c = 322.23
Resolution (Å)	30.0–2.9 (3.0–2.9)
Total observations	324008 (31401)
Unique reflections	54730 (5414)
Redundancy	5.9 (5.8)
R_{merge} (%)[a]	7.5 (56.0)
Completeness (%)	99.7 (100.0)
I/σ (I)	19.3 (3.1)

Values in parentheses show the statistics for the highest resolution shells.

[a] $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I_i \rangle|)/\Sigma_i |I_i|]$

3. Results and Discussion

SibL was overexpressed in *E. coli* with a yield of ~20 milligrams protein per liter of culture after purification. The purified protein contained an extra peptide, A_3LEH_6, at its C-terminus and was greater than 95% pure with a single band of approximately 38 kDa on SDS-PAGE. (Fig. 1). Using gel filtration analysis, we found that SibL exists as a dimer in solution. (Fig. 2)

SibL crystals were obtained by the sitting-drop vapor-diffusion method in a buffer comprising 50 mM HEPES buffer, pH 7.0, 40% (v/v) tacsimate, pH 7.0, 2 mM spermine and 2 mM hexamine cobalt (III) chloride. Well-diffracting crystals with dimensions of 0.1 × 0.1 × 0.05 mm were produced in 1 month (Fig. 3). Diffraction data were collected to a resolution limit of 2.9 Å (Fig. 4). The preliminary crystallographic analysis indicated that the crystal belonged to the space group *F*222, with unit-cell parameters *a* = 102.9, *b* = 295.4, *c* = 322.2 Å. Based on Matthews coefficient calculations, between seven (43% solvent content) and four (67% solvent content) molecules could be accommodated in the asymmetric unit, with an acceptable V_M in the range of 2.19-3.80 Å3 Da^{-1} (Kantardjieff & Rupp, 2003). Unfortunately, the structure of SibL could not be solved by molecular replacement, because we could not find any solved structure that share a sequence

identity higher than 25% with SibL. For resolving this difficulty, selenomethionyl SibL crystals have been produced with the goal of solving the structure by employing the multiwavelength anomalous diffraction (MAD) method. Crystallization and structure determination of SibL is the first step toward identifying the substrate-binding residues of this enzyme and gaining insight into its mechanism of action. Structure determination of selenomethionyl SibL is currently in progress.

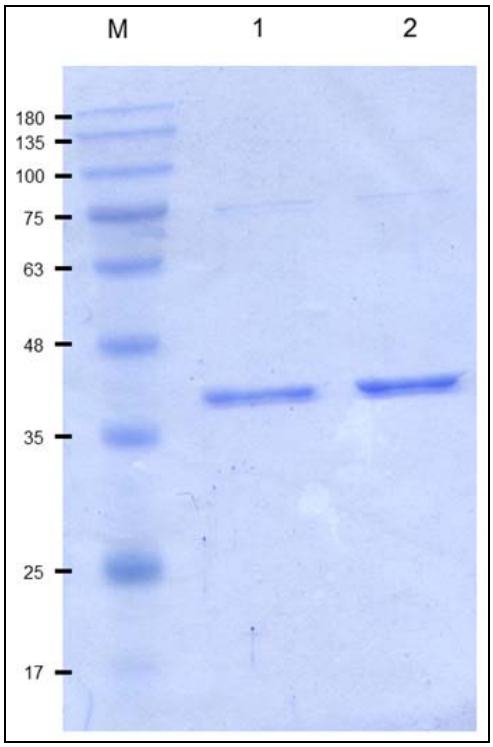

Fig 1: SDS-PAGE of purified SibL. Lane M, molecular-weight markers in kDa; lane 1, SibL purified with a Ni-column; lane 2, SibL purified by gel filtration.

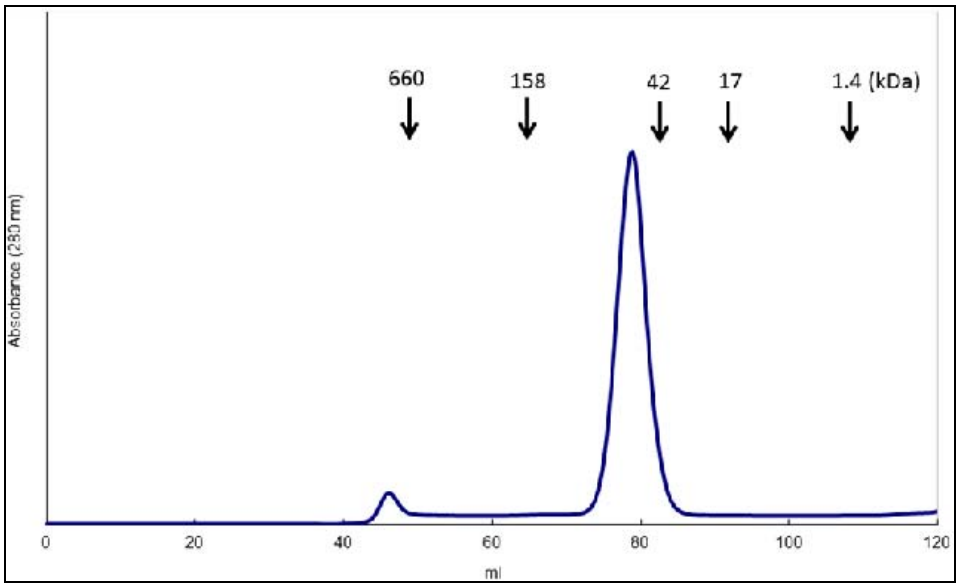

Fig 2: The size exclusion chromatography profile of SibL. The arrows indicates that molecular weight markers range from 1.4 to 660 kDa.

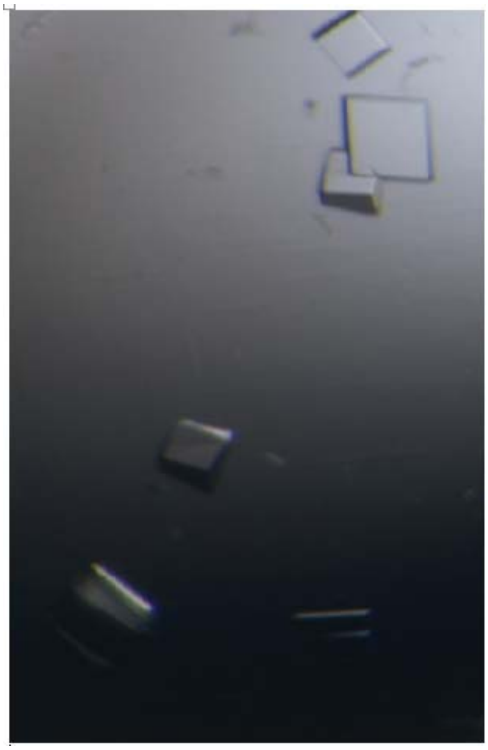

Fig 3: Crystals of SibL (dimensions 0.1 × 0.1 × 0.05 mm).

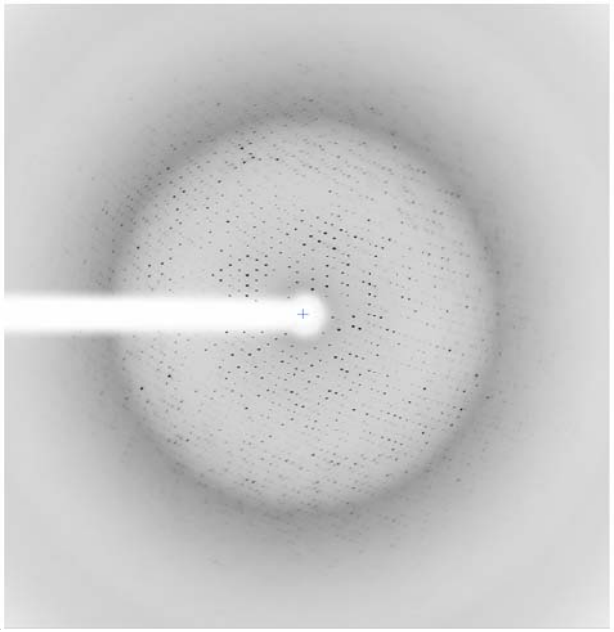

Fig 4: X-ray diffraction patterns recorded for SibL (1.0º oscillation)

4. Acknowledgments

We thank the National Synchrotron Radiation Research Center (NSRRC, Taiwan) for their assistance with regards to the data collection. We are also grateful to the staff of TCX-D900, Technology Commons, College of Life Science, and the Center for Systems Biology, NTU for help with the Phoenix protein crystallization robot (Art Robbins Instruments). This work was supported by grants from the National Science Council (NSC99-2313-B-241-001 and NSC100-2313-B-241-006 to Yeh Chen).

5. References

1. Clingen PH, De Silva IU, McHugh PJ, Ghadessy FJ, Tilby MJ, Thurston DE *et al.* The XPF-ERCC1 endonuclease and homologous recombination contribute to the repair of minor groove DNA interstrand crosslinks in mammalian cells produced by the pyrrolo [2, 1-c] [1, 4] benzodiazepine dimer SJG-136. Nucleic Acids Res 2005; 33:3283-3291
2. Crnovcic I, Sussmuth R, Keller U. Aromatic C-methyltransferases with antipodal stereoselectivity for structurally diverse phenolic amino acids catalyze the methylation step in the biosynthesis of the actinomycin chromophore. Biochemistry 2010; 49:9698-9705.

3. Gauze GF, Preobrazhenskaia TP, Ivanitskaia LP, Sveshnikova MA. Production of the antibiotic sibiromycin by the Streptosporangium sibiricum sp nov culture. Antibiotiki 1969; 14:963-969
4. Giessen TW, Kraas FI, Marahiel MA. A four-enzyme pathway for 3, 5-dihydroxy-4-methylanthranilic acid formation and incorporation into the antitumor antibiotic sibiromycin. Biochemistry 2011; 50:5680-5692.
5. Hurley LH, Thurston DE. Pyrrolo (l, 4) benzodiazepine Antitumor Antibiotics: Chemistry, Interaction with DNA, and Biological Implications. Pharm Res 1984; 1:52-59
6. Itoh J, Watabe H, Ishii S, Gomi S, Nagasawa M, Yamamoto H *et al.* A new pyrrolo [1, 4] benzodiazepine antitumor antibiotic produced by a Micromonospora sp. J Antibiot (Tokyo) 1988; 41:1281-1284.
7. Kantardjieff KA, Rupp B. Matthews coefficient probabilities: Improved estimates for unit cell contents of proteins, DNA, and protein-nucleic acid complex crystals. Protein Sci 2003; 12:1865-1871
8. Kopka ML, Goodsell DS, Baikalov I, Grzeskowiak K, Cascio D, Dickerson RE. Crystal structure of a covalent DNA-drug adduct: anthramycin bound to C-C-A-A-C-G-T-T-G-G and a molecular explanation of specificity. Biochemistry 1994; 33:13593-13610.
9. Kumar R, Lown JW. Recent developments in novel pyrrolo [2, 1-c] [1, 4] benzodiazepine conjugates: synthesis and biological evaluation. Mini Rev Med Chem 2003; 3:323-339.
10. Li W, Khullar A, Chou S, Sacramo A, Gerratana B. Biosynthesis of sibiromycin, a potent antitumor antibiotic. Appl Environ Microbiol 2009; 75:2869-2878.
11. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Macromolecular Crystallography Pt A 1997; 276: 307-326.
12. Wolters S, Neeb M, Berim A, Schulze Wischeler J, Petersen M, Heine A. Structural analysis of coniferyl alcohol 9-O-methyltransferase from Linum nodiflorum reveals a novel active-site environment. Acta Crystallogr D Biol Crystallogr 2013; 69:888-900.
13. Yonemoto IT, Li W, Khullar A, Reixach N, Gerratana B. Mutasynthesis of a potent anticancer sibiromycin analogue. ACS Chem Biol 2012; 7:973-977.