Purification, crystallization and preliminary X-ray diffraction studies of UDP-glucose: tetrahydrobiopterin α -glucosyltransferase (BGluT) from *Synechococcus* sp. PCC 7942

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A UDP-glucose:tetrahydrobiopterin α -glucosyltransferase (BGluT) enzyme was discovered in the cyanobacterium *Synechococcus* sp. PCC 7942 which transfers a glucose moiety from UDP-glucose to tetrahydrobiopterin (BH₄). BGluT protein was overexpressed with selenomethionine labelling for structure determination by the multi-wavelength anomalous dispersion method. The BGluT protein was purified by nickel-affinity and size-exclusion chromatography. It was then crystallized by the hanging-drop vapour-diffusion method using a well solution consisting of 0.1 M bis-tris pH 5.5, 19% (w/v) polyethylene glycol 3350 with 4% (w/v) D(+)-galactose as an additive. X-ray diffraction data were collected to 1.99 Å resolution using a synchrotron-radiation source. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 171.35$, $b = 77.99$, $c = 53.77$ Å, $\beta = 90.27^\circ$.

1. Introduction

Pteridine glycosyltransferases (PGTs) are a group of enzymes which catalyze the transfer of various glycosyl groups to a set of reduced-form pteridine compounds including L-erythro-tetrahydrobiopterin (BH₄; Chung *et al.*, 2000). Tetrahydrobiopterin (BH₄) is an indispensable cofactor for aromatic amino-acid hydroxylases and nitric oxide synthases in animals and has been implicated in neuronal disorders in humans (Hashimoto *et al.*, 2004; Werner *et al.*, 2011). Interestingly, glycosidic forms of BH₄ have been found in archaea, cyanobacteria (Chung *et al.*, 2000; Wachi *et al.*, 1995) and species of the anaerobic photosynthetic bacteria *Chlorobium* (Cho *et al.*, 1998; Kang *et al.*, 1998).

PGTs belong to a novel group of glycosyltransferases (GTs) amongst the 94 glycosyltransferase families classified by the CAZY classification system (<http://www.cazy.org>). GT structures determined to date adopt one of three folds: GT-A, GT-B and GT-A-like (Lairson *et al.*, 2008). From amino-acid sequence analysis of PGTs they are predicted to adopt the GT-B fold with two domains, of which the N-terminal domain is responsible for acceptor binding and the C-terminal domain is responsible for glycoside binding. GTs have been studied intensively for the glycodiversification of therapeutically important natural compounds. Interestingly, PGTs could be applicable to the quantification of reduced and oxidized forms of BH₄ and possibly for BH₄ glucoside production (Kim *et al.*, 2010). One of the PGTs found in the cyanobacterium *Synechococcus* sp. PCC 7942, named UDP-glucose:tetrahydrobiopterin α -glucosyltransferase (BGluT; UniProt ID Q93EY3), catalyzes the synthesis of BH₄-glucoside from UDP-glucose and BH₄ (Chung *et al.*, 2000; Choi *et al.*, 2001; Hwang *et al.*, 2002; Cha *et al.*, 2005). The biosynthesis and biological functions of pteridine glucoside are not well understood. Here, we report the purification, crystallization and preliminary diffraction analysis of selenomethionine (SeMet)-labelled BGluT protein. The structure of BGluT would provide a detailed explanation of PGT enzyme catalysis and its specificities toward various sugar donors for the synthesis of pteridine glucoside.

2. Materials and methods

2.1. Cloning

The BGluT gene was amplified by PCR from genomic DNA of *Synechococcus* sp. PCC 7942 with the forward primer 5'-CGACCA-TGGATGCCCCACCGTTTTCTG-3' and the reverse primer 5'-GC-GCTCGAGCTAGAAGCCTCGGGCTCGGGA-3' containing *NcoI* and *XhoI* restriction-enzyme sites (bold), respectively. To correct the reading-frame mismatch arising from the *NcoI* restriction site, AT was added after the *NcoI* site in the forward primer, which resulted in the replacement of the second residue Thr by Asp in the BGluT sequence. The amplified gene was cloned into a pProEX HTa vector (Life Technologies, Carlsbad, California, USA) for the over-expression of BGluT with a hexahistidine tag (underlined), a TEV cleavage site (bold) and residues MD (italics) instead of MT at the N-terminus (MSYYHHHHHHHDYDIPTTENLYFQGAMD) in *Escherichia coli*. The vector construct was verified by DNA sequencing.

2.2. Protein expression and purification

The cloned plasmid pProEX HTa carrying the gene fragment of BGluT was transformed into *E. coli* strain BL21(DE3) for expression. A single colony harbouring the vector was grown overnight in 100 ml Luria-Bertani medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre of solution) at 310 K in the presence of ampicillin (100 mg ml⁻¹). 20 ml of the overnight culture was used to inoculate 1000 ml M9 medium (6 g Na₂HPO₄, 3 g KH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl, 2 g glucose, 2 mM MgSO₄, 0.1 mM CaCl₂ per litre of Milli-Q water) with ampicillin (100 mg ml⁻¹). 40 mg ml⁻¹ selenomethionine (Acros Organics, New Jersey, USA) was added together with 17 other solid L-amino acids (BioShop Canada Inc., Burlington, Canada). The cells were grown at 310 K until the OD₆₀₀ reached 0.6 and protein expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 303 K overnight. The cells were harvested by centrifugation at 6520g at 277 K for 10 min. The cell pellet was resuspended in 80 ml binding/lysis buffer consisting of 50 mM phosphate pH 8.0, 500 mM NaCl with 5 mM β-mercaptoethanol and was disrupted by sonication. It was then centrifuged at 15 930g and 277 K for 30 min. The supernatant was collected and filtered (qualitative filter paper; Advantec, Japan).

The filtered supernatant was poured into a nickel-agarose affinity column (Qiagen, Hilden, Germany) that had been pre-equilibrated with the binding/lysis buffer. The column was washed with two column volumes of washing buffer consisting of 50 mM phosphate pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 30 mM imidazole. The protein was eluted with elution buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 300 mM imidazole, 5 mM β-mercaptoethanol. The eluted fractions were examined by SDS-PAGE. The fractions containing BGluT were pooled, concentrated and exchanged into buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol by ultrafiltration (Centricon YM-30; Millipore, Bedford, Massachusetts, USA). The BGluT protein was then treated with TEV protease in a 1:20(w:w) ratio overnight at 277 K to cleave the His tag. By running SDS-PAGE, it was confirmed that the TEV cleavage was complete and successful. After TEV cleavage, Gly and Ala residues remained fused to the N-terminus of BGluT. The TEV-treated BGluT proteins were applied onto a nickel-agarose affinity column (Qiagen, Hilden, Germany) to remove TEV protease and uncleaved proteins. The flowthrough fractions were collected and applied onto a Superdex 200 gel-filtration column (GE Healthcare, Piscataway, New Jersey, USA) equilibrated with 20 mM Tris-HCl pH

8.0, 150 mM NaCl, 1 mM DTT in an FPLC system (GE Healthcare). The BGluT proteins separated by gel filtration were concentrated to 10 mg ml⁻¹ by ultrafiltration (Centricon YM-30; Millipore) for crystallization. All purification steps were performed with ice-cooled buffers at room temperature, which we believe help to keep the protein stable. Protein purity was monitored by SDS-PAGE and native PAGE. Protein concentration was determined by the Bradford assay (Bradford, 1976; Zor & Selinger, 1996) using bovine serum albumin as a standard.

2.3. Crystallization

Crystallization of SeMet-labelled BGluT protein was initially carried out with Crystal Screen, Crystal Screen 2 and Index (Hampton Research, California, USA), Wizard Screens I and II and Cryo I and II (Emerald BioStructures, Bainbridge Island, Washington, USA) and laboratory-made screening solutions using a microbatch crystallization method at 291 K with Al's oil (D'Arcy *et al.*, 2003). Drops were prepared with a mixture of equal volumes of protein solution (1 µl) and screening solution (1 µl) and equilibrated under Al's oil in 72-well plates. Among the screening solutions, microcrystals were initially found from Index condition No. 42 [0.1 M bis-tris pH 5.5, 25%(w/v) polyethylene glycol 3350]. The crystallization condition was optimized to obtain better crystals using the hanging-drop vapour-diffusion method, varying the buffer and precipitant concentrations. An optimized reservoir solution consisting of 0.1 M bis-tris pH 5.5, 19%(w/v) PEG 3350 was found to produce good microcrystals. Additives were screened using Additive Screen (Hampton Research, California, USA) to improve the crystal size. For additive screening, 1 µl additive and 9 µl reservoir solution were mixed, and 1 µl of this mixed solution was combined with 1 µl BGluT protein (10 mg ml⁻¹) in a single drop. Larger BGluT crystals were grown using Additive Screen condition No. 60 [30%(w/v) D(+)-galactose]. The condition was then further optimized by changing the concentration of galactose directly in the reservoir solution using the hanging-drop vapour-diffusion method. The best crystals were obtained using a well solution consisting of 0.1 M bis-tris pH 5.5, 19%(w/v) PEG 3350, 4%(w/v) D(+)-galactose. Rod-shaped crystals appeared in a couple of days and grew to 0.3 mm in length (Fig. 1).

2.4. X-ray data collection

For X-ray diffraction experiments, the crystals were quickly transferred into a cryosolution consisting of 0.1 M bis-tris pH 5.5,

Figure 1
A BGluT protein crystal obtained by the hanging-drop vapour-diffusion method. A rod-shaped single crystal formed using a well solution consisting of 0.1 M bis-tris pH 5.5, 19%(w/v) PEG 3350 with 4%(w/v) D(+)-galactose as an additive.

Figure 2

An X-ray diffraction image from a BGluT protein crystal. The black circle corresponds to 1.99 Å resolution. The inset represents a magnified view of the area at the edge close to 1.99 Å resolution indicated in the square.

19%(w/v) PEG 3350, 25%(v/v) glycerol. After a short soak, the crystals were picked up with cryoloops and flash-cooled in liquid nitrogen. X-ray diffraction data were collected from the rod-shaped BGluT crystals on beamlines 17A at the Photon Factory (PF), Japan and 5C at the Pohang Accelerator Laboratory (PAL), Republic of Korea. Three data sets were collected from a single crystal at peak (0.97919 Å), edge (0.97951 Å) and remote (0.96427 Å) wavelengths at 100 K. For each data set, 220 diffraction images were collected with an oscillation angle of 1°, an exposure of 1 s and a crystal-to-detector distance of 250 mm. All diffraction images were indexed, integrated and scaled using the *HKL-2000* suite (Otwinowski & Minor, 1997). Data statistics are shown in Table 1.

3. Results and discussion

The BGluT gene was cloned successfully in pProEx HTa expression vector and was expressed in soluble form in *E. coli* BL21(DE3) cells. The BGluT protein was purified by nickel-affinity chromatography and size-exclusion (Superdex 200) chromatography. The yield of pure protein was about 0.5 mg per litre of culture. The size-exclusion chromatography profile indicated that the protein was a monomer with an apparent molecular weight of ~40 kDa, matching the theoretical value calculated from the amino-acid sequence (359 amino acids). Multiple anomalous dispersion data sets were collected from selenomethionine-labelled BGluT crystals, which diffracted to 1.99 Å resolution (Fig. 2). The space group was determined to be the monoclinic space group *C2*, with unit-cell parameters $a = 171.35$, $b = 77.99$, $c = 53.77$ Å, $\beta = 90.27^\circ$. Because the β angle was so close to 90° ($\beta = 90.27^\circ$), scaling was first attempted in the orthorhombic space group *C222*, but this space group was clearly incorrect. From the analysis in space group *C2*, the Matthews coefficient is $2.3 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968) and the solvent content is 46.54%,

Table 1

Crystal data statistics for apo SeMet BGluT protein.

Values in parentheses are for the highest resolution shell.

	Peak	Edge	Remote
Space group	<i>C2</i>		
Unit-cell parameters (Å, °)	$a = 171.35$, $b = 77.99$, $c = 53.77$, $\alpha = \gamma = 90$, $\beta = 90.27$		
Wavelength (Å)	0.97919	0.97951	0.96427
Resolution (Å)	50–1.99	50–1.99	50–1.99
	(2.02–1.99)	(2.02–1.99)	(2.02–1.99)
Unique reflections	48642 (2380)	48577 (2363)	48827 (2397)
Completeness (%)	99.9 (99.9)	100 (100)	100 (99.8)
$R_{\text{merge}}^\dagger$ (%)	8.3 (55)	8.6 (68)	10.2 (92)
Multiplicity	4.6 (4.5)	4.7 (4.5)	4.6 (4.5)
$\langle I/\sigma(I) \rangle$	8.6 (3.1)	7.8 (2.7)	6.7 (2.0)
No. of molecules in asymmetric unit	2		
Matthews coefficient ($\text{Å}^3 \text{ Da}^{-1}$)	2.3		
Solvent content (%)	46.54		
Figure of merit	0.36/0.77‡		

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl . ‡ Figure of merit before and after density modification.

assuming that there are two molecules in the asymmetric unit. No structure with high sequence homology suitable for determining the structure of BGluT by molecular replacement is available in the Protein Data Bank. Instead, the structure of BGluT was determined by SAD phasing using *PHENIX* (Adams *et al.*, 2010). Eight out of ten Se atoms (five selenomethionines are present in each molecule) were found, from which initial electron density was obtained that was suitable for model building. Model building and refinement are under way. A detailed discussion of the structure determination will be published elsewhere.

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