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Expression, purification, crystallization and preliminary X-ray analysis of sepiapterin reductase from *Chlorobium tepidum*

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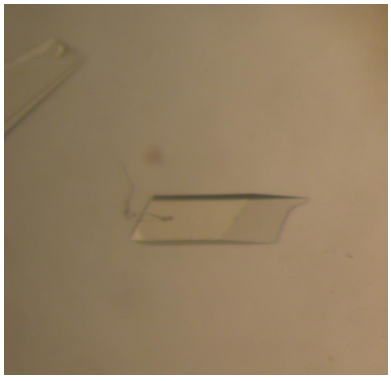

Sepiapterin reductase from *Chlorobium tepidum* (CT-SR) produces L-*threo*-tetrahydrobiopterin, an isomer of tetrahydrobiopterin, in the last step of *de novo* synthesis initiating from GTP. Native CT-SR and a selenomethionine (SeMet) derivative of CT-SR have been crystallized by the hanging-drop vapour-diffusion method using PEG 400 as precipitant. CT-SR crystals belong to space group *R*32, with unit-cell parameters $a = b = 201.142$, $c = 210.184$ Å, and contain four molecules in the asymmetric unit. Diffraction data were collected to 2.1 Å resolution using synchrotron radiation. The structure of CT-SR has been determined using MAD phasing. There is one CT-SR tetramer in the asymmetric unit formed by two closely interacting CT-SR dimers. The solvent content is calculated to be about 67.2%.

1. Introduction

Sepiapterin reductase (SR; EC 1.1.1.153) catalyzes the last step of the *de novo* synthesis of tetrahydrobiopterin (BH_4) from GTP. BH_4 is a multifunctional cofactor that is involved in a variety of important physiological processes in humans and other higher organisms (reviewed in Thöny *et al.*, 2000; Werner-Felmayer *et al.*, 2002). It is a well known essential cofactor for aromatic amino-acid hydroxylases (Kaufman, 1993) and nitric oxide synthase (NOS; Marletta, 1994). It has been proposed to be involved in proliferation of murine erythrocyte cells (Tanaka *et al.*, 1989), promotion of neurotransmitter release in the brain (Magata *et al.*, 1991) and regulation of human melanogenesis (Schallreuter *et al.*, 1994). BH_4 deficiency causes severe neurological disorders characterized by hyperphenylalaninaemia and monoamine neurotransmitter deficiency and is also implicated in Parkinson's disease, Alzheimer's disease and depression (Blau *et al.*, 2002; Thöny *et al.*, 2000). BH_4 can also be found in lower eukaryotes including fungi (Maier & Ninnemann, 1995) and in particular groups of bacteria such as cyanobacteria (Chung *et al.*, 2000) and anaerobic photosynthetic bacteria *Chlorobium* species (Cho *et al.*, 1998) as glycosidic forms.

The pathway of the *de novo* biosynthesis of BH_4 from GTP involves only three enzymes: GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), 6-pyruvoyl-tertrahydropterin (PPH_4) synthase (PTPS; EC 4.2.3.12) and SR. SR reduces the diketo group in the C6 side chain of PPH_4 to BH_4 in an NADPH-dependent manner (Katoh & Sueoka, 1984) (Fig. 1). Based on biochemical and crystallographic data from mouse sepiapterin reductase (mSR), it has been proposed that SR first reduces the C1′ carbonyl group in an NADPH-dependent manner and subsequently catalyzes an isomerization reaction shifting the C2′ carbonyl group to the C1′ position; it then catalyzes the second NADPH-dependent reduction of the carbonyl group to

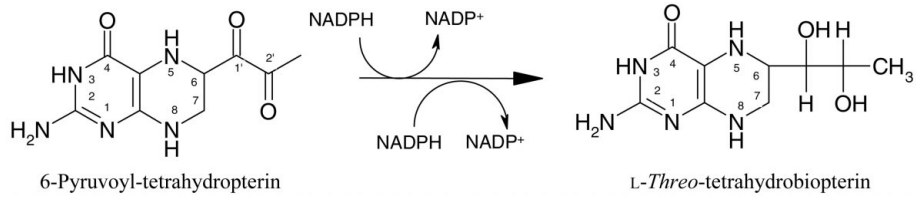

Figure 1
Enzymatic reaction catalyzed by sepipaterin reductase from *C. tepidum*.

produce L-*erythro*-BH_4 (Katoh & Sueoka, 1988; Auerbach *et al.*, 1997). Although L-*erythro*-BH_4 [6*R*-(1′*R*,2′*S*)-5,6,7,8-BH_4] is common in nature, other stereoisomers such as D-*threo*-6*R*-(1′*R*,2′*R*)- and L-*threo*-6*R*-(1′*S*,2′*S*) are also found. The D-*threo* form (dictyopterin) has been found in *Dictyostelium discoideum* (Klein *et al.*, 1990). A glycosylated L-*threo* stereoisomer has been isolated from *Chlorobium tepidum* (tepidopterin, L-*threo*-BH_4-*N*-acetylglucosamine; Cho *et al.*, 1998). It has been shown that L-*threo*-BH_4 is produced from PPH_4 by *C. tepidum* SR (CT-SR; Choi *et al.*, 2005).

CT-SR has high protein-sequence similarities with the mammalian SRs including mouse SR (40% similarity). CT-SR is predicted to share the same fold as the homologous mouse SR (mSR) protein in its substrate- and cofactor-binding sites (Auerbach *et al.*, 1997). However, CT-SR catalyzes the synthesis of L-*threo*-BH_4, while mSR only catalyzes the synthesis of L-*erythro*-BH_4 from PPH_4.

In this work, we describe the expression, purification, crystallization and preliminary diffraction data of CT-SR. Knowledge of the three-dimensional structure of the active site of CT-SR will provide a deeper understanding of the catalytic mechanism of the enzyme activity in comparison with the mSR structure.

2. Materials and methods

2.1. Protein expression and purification

The CT-SR gene was cloned into a pET-28b (Novagen, Madison, WI, USA) expression plasmid to produce recombinant CT-SR with histidine tags. The plasmid was transformed into *Escherichia coli* strain BL21(DE3) for protein expression (Choi *et al.*, 2005). 10 ml aliquots of an overnight culture were subcultured into 1000 ml fresh LB (Luria–Bertani) medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre of solution) plus ampicillin (100 µg ml^{-1}) and allowed to grow to OD_{600} = 0.6 at 300 K. Protein expression was induced for 3 h with 0.4 m*M* isopropyl-β-D-thiogalactoside and cells were harvested by centrifugation (6 min, 1000 rev min^{-1}). The harvested cells were washed with lysis buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 5 m*M* imidazole), resuspended in the same buffer and disrupted by sonication. After centrifugation (1 h, 12 000 rev min^{-1}) at 277 K, the clear supernatant was filtered (pore diameter 0.45 µm; Satorius, Goettingen, Germany) and applied onto a column of nickel–NTA beads (Quiagen, Hilden, Germany) pre-equilibrated with lysis buffer. The column was washed first with 20 column volumes of lysis buffer and then with 20 column volumes of 50 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl, 30 m*M* imidazole. The recombinant CT-SR was eluted with 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 300 m*M* imidazole. Fractions containing CT-SR were pooled, concentrated and exchanged to 50 m*M* Tris–HCl pH 8.0 by ultrafiltration (Centriprep YM-30, Millipore Corporation, Bedford, MA, USA). The CT-SR was further purified by anion-exchange chromatography (MonoQ, Amersham Biosciences Limited, UK). The protein was eluted with a salt gradient and separated at ∼0.3 *M* NaCl pH 8.0. The fractions containing CT-SR were concentrated by ultrafiltration (Centriprep YM-30, Milipore Corporation, Bedford, MA, USA). The CT-SR was finally purified by gel-filtration chromatography with a Superdex 200 column (Amersham Biosciences Ltd, UK) in 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl and concentrated to a final concentration of 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 by ultrafiltration (Microcon YM-30, Millipore Corporation, Bedford, MA, USA). The protein purity was examined by SDS–PAGE. The protein concentration was determined by Bradford assay. To facilitate phase determination, CT-SR proteins containing selenomethionines (SeMet) substituted for ten methionine residues were expressed and purified in the same way as the native CT-SR.

2.2. Crystallization and data collection

Crystallization was initially carried out with Crystal Screens I, II and Index (Hampton Research, CA, USA) and Wizard Screens I, II, Cryo I and II (Emerald BioStructures, Bainbridge Island, WA, USA) using a microbatch crystallization method at 291 K. Drops containing equal volumes (1 µl) of protein (10 mg ml^{-1}) and the screening solution were equilibrated under Al's oil in a 72-well microbatch plate. Screening solution No. 25 (0.2 *M* $MgCl_2$, 0.1 *M* Tris pH 8.5, 30% PEG 400) from Wizard I produced rod-shaped microcrystals. Further screenings to find optimal crystallization conditions for crystal growth were accomplished by hanging-drop vapour-diffusion trials varying the salt and precipitant concentrations and the volume of the drop. The best large crystals were obtained at 291 K in a drop containing 4 µl 10 mg ml^{-1} protein solution (20 m*M* Tris–HCl pH 8.0) and 1 µl of a mixture of 4 µl reservoir solution (0.2 *M* $MgCl_2$, 0.1 *M* Tris–HCl pH 8.5, 34% PEG 400) and 1 µl additive (1 *M* guanidine hydrochloride). Crystals grew to maximum dimensions of 1.0 × 0.3 × 0.3 mm in 2 d (Fig. 2). The SeMet protein crystallizes isomorphously with the native enzyme under the same conditions. To prepare a CT-SR–NADP–sepiapterin complex and a CT-SR–NADP–*N*-acetylserotonin complex, crystals of native enzyme were soaked in reservoir solution containing 1 m*M* NADP and 1 m*M* sepiapterin and containing 1 m*M* NADP and 1 m*M* *N*-acetylserotonin, respectively. Crystals were flash-frozen in liquid nitrogen for data collection after soaking for 30 min in the reservoir solution.

X-ray diffraction data for native CT-SR were collected from a single crystal to 2.1 Å resolution using X-rays of wavelength 1.1273 Å and a Bruker CCD detector at station 6B of the Pohang Accelerator Laboratory, Pohang, Korea. The crystal-to-detector distance was set to 230 mm and a 1° oscillation and 30 s exposure were used per image. Diffraction data for the SeMet derivative were collected from a single crystal to 2.1 Å resolution at station 6A at the Photon Factory at the High Energy Accelerator Research Organization, Tsukuba, Japan. A total of 180 images were collected with 1° oscillation and 40 s exposure per frame at wavelengths of 0.978 Å (peak), 0.97934 Å (edge) and 0.97035 Å (remote). All diffraction images were indexed, integrated and scaled using the *HKL*2000 suite (Otwinowski & Minor, 1997). Data statistics are shown in Table 1.

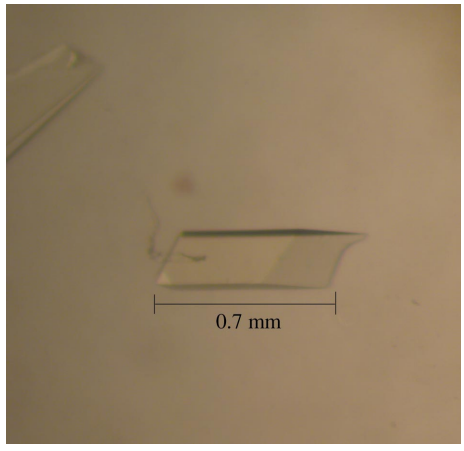

Figure 2
Crystals of sepiapterin reductase from *C. tepidum*, with approximate dimensions 0.7 × 0.3 × 0.3 mm.

Table 1
Data statistics for CT-SR crystals.

Values in parentheses are for the highest resolution shell.

		SeMet		
	Native	Peak	Edge	Remote
Space group	*R*32			
Unit-cell parameters (Å)	a = 202.143, b = 202.143, c = 210.018			
No. of chains in AU	4			
V_M (Å^3 Da^{-1})	3.8			
Solvent content (%)	67.2			
Wavelength (Å)	1.123	0.978	0.97934	0.097035
Resolution (Å)	50–2.15 (2.23–2.15)	25–2.1 (2.18–2.1)	25–2.1 (2.18–2.10)	25–2.1 (2.18–2.1)
Unique reflections	88395 (8730)	94700 (9360)	94812 (9379)	94880 (9396)
Completeness (%)	99.2 (98.5)	99.9 (99.7)	99.9 (99.7)	99.9 (99.7)
R_{merge}† (%)	14.6 (89.7)	11.3 (60.2)	10.4 (64)	10.9 (66.7)
Redundancy	19	10.2	10.3	10.1
$I/\sigma(I)$	7.0	11.1	11.0	11.6
Figure of merit (before/after solvent treatment)			0.47/0.68	
Overall Z score (*SOLVE*)‡			98.5 (for 33 Se sites)	

† $R_{merge} = \sum |I(h) - \langle I(h)\rangle| / \sum I(h)$, where $I(h)$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$. ‡ *SOLVE* uses the 10–30 seeds generated from the analyses of Patterson functions as trial solutions and scores each seed. From this set of scores, an average score and the standard deviation of this average score is obtained for each criteria. A Z score (number of standard deviations above the mean) is then calculated for each trial solution for each criteria. The overall Z score for a solution is the sum of the individual Z scores corrected for any large deviation among the scores. A good solution will have a high Z score (typically above 20; this score is higher for structures with more heavy atoms).

3. Results

A 2.1 Å resolution data set was collected and processed from a native CT-SR crystal. The space group was found to be rhombohedral *R*32, with unit-cell parameters $a = b = 202.143$, $c = 210.018$ Å (Table 1). The molecular weight of the protein had been calculated to be about 55 kDa from gel-filtration chromatography and the monomer of CT-SR to be approximately 26 kDa from SDS–PAGE. Consequently, the protein exists as a dimer in solution. Attempts to solve the structure by molecular replacement using the program *AMoRe* (Navaza & Vernoslova, 1995) with the mouse sepiapterin reductase (PDB code 1sep; Auerbach *et al.*, 1997) as a search model have given no useful solutions. Thus, experimental phasing was attempted by the multiple-wavelength anomalous dispersion (MAD) method based on the SeMet derivative, which crystallized isomorphously with the native crystals under the same conditions. Diffraction was recorded at peak, inflection and high-energy remote points (Table 1). 33 selenium sites were located and phases were calculated using *SOLVE* (Terwilliger & Berendzen, 1999). According to the specific volume calculations (Matthews, 1968) based on the unit-cell parameters and the molecular weight, it appeared that there could be six or eight molecules per asymmetric unit, with solvent contents of 50.8 and 34.4% (V_M = 2.5 and 1.9 Å^3 Da^{-1}), respectively. However, electron densities calculated with MAD phases unexpectedly revealed there to be four molecules in the asymmetric unit, with a solvent content of 67.2% and a specific volume V_M of 3.8 Å^3 Da^{-1} (Table 1). A CT-SR tetramer is formed by close interaction of two dimers in the asymmetric unit (Fig. 3). The structure of CT-SR has been determined using the MAD phases and will be published elsewhere. Structure determinations for the CT-SR–NADP–sepiapterin and CT-SR–NADP–*N*-acetylserotonin complexes are in progress.

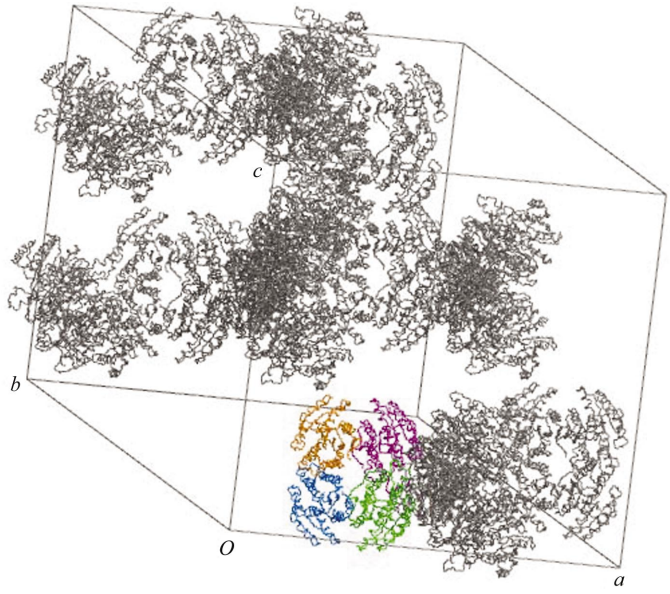

Figure 3
A tetramer of CT-SR formed by two dimers in the asymmetric unit. All molecules in the unit cell are displayed in black, except for one tetramer in the AU with chains in orange, green, cyan and magenta.

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