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Crystallization and X-ray analysis of 2-deoxy-scylo-inosose synthase, the key enzyme in the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics

A recombinant 2-deoxy-scylo-inosose synthase from *Bacillus circulans* has been crystallized at 277 K using PEG 4000 as precipitant. The diffraction pattern of the crystal extends to 2.30 Å resolution at 100 K using synchrotron radiation at the Photon Factory. The crystals are monoclinic and belong to space group $P2_1$, with unit-cell parameters $a = 80.5$, $b = 70.4$, $c = 83.0$ Å, $\beta = 117.8^\circ$. The presence of two molecules per asymmetric unit gives a crystal volume per protein weight (V_M) of $2.89 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent constant of 57.4% by volume.

1. Introduction

2-Deoxystreptamine-containing aminoglycoside antibiotics, including neomycin, kanamycin, gentamicin and ribostamycin, are clinically important antibacterial agents (Rinehart & Stroshane, 1976; Rinehart & Suami, 1980). A key enzyme in the biosynthesis of 2-deoxystreptamine (DOS) is 2-deoxy-scylo-inosose synthase (DOIS), which catalyzes the multistep cyclization of D-glucose-6-phosphate (G-6-P) into the six-membered carbocycle 2-deoxy-scylo-inosose in the first step of DOS biosynthesis (Fig. 1; Daum *et al.*, 1977; Furumai *et al.*, 1979; Kakinuma *et al.*, 1981, 1989; Yamauchi & Kakinuma, 1992, 1993, 1995; Kudo *et al.*, 1997; Rinehart & Stroshane, 1976).

We report the first isolation and functional characterization of DOIS from butirosin-producing *Bacillus circulans* SANK72073 by cloning the gene (*btrC*) and overexpressing it in *Escherichia coli* (Kudo, Hosomi *et al.*, 1999; Kudo, Tamegai *et al.*, 1999). A certain amount of homology (25–33%) was observed at the amino-acid level between the *B. circulans* DOIS and dehydroquinase synthase (DHQS) derived from several microbes. DHQS, an enzyme in the shikimate pathway, catalyzes the cyclization of 3-deoxy-D-arabino-heptulosonate-7-phosphate to dehydroquinase in a similar manner to the DOIS reaction. Despite these similarities, significant differences have been demonstrated. An important difference is found in the stereochemistry of the overall reaction. In the DHQS reaction, the phosphate elimination takes place in *syn* fashion by intramolecular self-catalysis. On the other hand, *anti*-elimination of the phosphate is postulated for the DOIS reaction (Nango *et al.*, 2003,

Figure 1
Reaction mechanism of DOIS.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 80.5$, $b = 70.4$, $c = 83.0$, $\beta = 117.8$
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.89
Resolution ($\text{\AA}$)	24.77–2.30 (2.42–2.30)
Total observations	353773
Unique reflections	36626
$I/\sigma(I)$	5.6 (3.0)
$R_{\text{merge}}^\dagger$ (%)	11.3 (26.9)
Data completeness (%)	99.8 (100)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , including symmetry-related reflections, and $\langle I(hkl) \rangle$ is its average.

2004). Since the crystallographic structures of various DHQS enzymes have been elucidated (Carpenter *et al.*, 1998; Nichols *et al.*, 2003; Nichols, Hawkins *et al.*, 2004; Nichols, Ren *et al.*, 2004; Sugahara *et al.*, 2005), it is extremely intriguing to figure out the structural similarities and dissimilarities of this class of enzymes. In addition, we have recently demonstrated a promising short-step conversion of D-glucose into catechol by a chemoenzymatic approach with the over-expressed BtrC (Kakinuma *et al.*, 2000).

In this study, we report the crystallization and preliminary X-ray diffraction analysis of BtrC.

2. Experimental

2.1. Expression and purification

The expression of BtrC was carried out according to the method described by Kudo, Tamegai *et al.* (1999). The purification was performed by the procedure adopted in the previous method. After chromatographic purification with a Hi-Load 26/60 Superdex 200pg column (Pharmacia Biotech) on FPLC (Pharmacia Biotech), the fractions containing BtrC were combined and concentrated to approximately 2 ml by centrifugation with a Vivaspin 20 ml concentrator (Satorius AG, Germany). The concentrate was then loaded onto a Hi-Load 16/10 phenyl Sepharose HP column (Pharmacia Biotech) previously equilibrated with 5 mM Tris–HCl pH 7.7

containing 1 M $(\text{NH}_4)_2\text{SO}_4$ and 0.2 mM CoCl_2 and the column was washed with 40 ml of the same buffer. The adsorbed proteins were then eluted with 5 mM Tris–HCl pH 7.7 containing 0.2 mM CoCl_2 with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ concentration (1–0 M) for 30 min at 2 ml min^{−1}. BtrC was eluted in the fractions of approximately 0 M $(\text{NH}_4)_2\text{SO}_4$. The purified enzyme was replaced by 5 mM Tris–HCl pH 7.7 containing 0.2 mM CoCl_2 , concentrated to 10 mg ml^{−1} and stored at 193 K.

2.2. Crystallization

BtrC crystals were grown by the hanging-drop vapour-diffusion method at 277 K. Initial screening was performed using Crystal Screen reagent kits (Hampton Research). Several crystal forms were obtained and one of the better crystallization conditions was optimized. A 2 μ l droplet of 4.2 mg ml^{−1} protein solution mixed with the same amount of reservoir solution was equilibrated against 1 ml reservoir solution [40% (w/v) PEG 4000, 200 mM Li_2SO_4 and 100 mM Tris–HCl pH 8.6] to obtain BtrC crystals.

2.3. Data collection

Crystals were soaked for a few seconds in a solution containing 40% (w/v) PEG 4000, 200 mM Li_2SO_4 , 100 mM Tris–HCl pH 8.6 and 20% (v/v) glycerol. The crystal mounted in a cryoloop was flash-cooled in a liquid-nitrogen stream at 100 K. Data collection was performed at 100 K using a wavelength of 0.97 $\text{\AA}$ from the synchrotron-radiation source at the Photon Factory BL6A station (Tsukuba, Japan) with an ADSC Quantum 4R CCD detector system (ADSC, California, USA). The data were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994).

Figure 2

Crystals of BtrC. The average dimensions of these crystals were $0.1 \times 0.1 \times 0.3$ mm.

Figure 3

Diffraction pattern of a BtrC crystal. The enlarged image of the area around 2.30 $\text{\AA}$ is shown.

3. Results and discussion

The crystallization conditions for recombinant BtrC were obtained by the sparse-matrix method and were optimized to produce a single crystal suitable for X-ray analysis. Crystals grew to maximum dimensions of $0.1 \times 0.1 \times 0.3$ mm (Fig. 2). The crystals produced diffraction data to 2.30 Å resolution at the Photon Factory BL6A station (Fig. 3). From the diffraction data collected, the space group was determined to be monoclinic $P2_1$, with unit-cell parameters $a = 80.5$, $b = 70.4$, $c = 83.0$ Å, $\beta = 117.8^\circ$. Assuming one dimeric subunit in the asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) was calculated to be $2.89 \text{ Å}^3 \text{ Da}^{-1}$ and the estimated solvent content is 57.4%.

Scaling and merging of the crystallographic data resulted in an overall R_{merge} of 6.3% and an R_{merge} in the highest resolution shell of 26.9%. Complete data-collection statistics are given in Table 1.

BtrC shows sequence homology to several DHQS enzymes of known structure. We have attempted to carry out molecular replacement for phase determination, but have not succeeded. The crystal structure is now being solved by the MIRAS method using several heavy-atom derivatives.

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