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Crystallization and preliminary crystallographic analysis of the catechol 2,3-dioxygenase PheB from *Bacillus stearothermophilus* BR219

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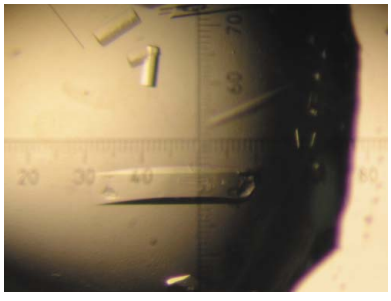

Class II extradiol-cleaving catecholic dioxygenase, a key enzyme of aromatic compound degradation in bacteria, cleaves the aromatic ring of catechol by adding two O atoms. PheB is one of the class II extradiol-cleaving catecholic dioxygenases and shows a high substrate specificity for catechol derivatives, which have one aromatic ring. In order to reveal the mechanism of the substrate specificity of PheB, PheB has been crystallized by the hanging-drop vapour-diffusion method using PEG 4000 as a precipitant. The space group of the obtained crystal was $P2_12_12_1$, with unit-cell parameters a = 65.5, b = 119.2, c = 158.7 Å. The crystal diffracted to 2.3 Å resolution.

1. Introduction

Extradiol-cleaving catecholic dioxygenases (ECDOs; EC 1.13.11.2) play a key role in the degradation pathway of aromatic compounds. To date, various types of ECDOs have been cloned from bacteria such as pseudomonads (Fukuda, 1993). These enzymes catalyze the addition of two O atoms to the catechol ring of the substrate, resulting in cleavage of the catechol ring. ECDOs typically contain a non-haem iron (Fe^{2+}) in the active site (Solomon *et al.*, 2000; Bugg, 2003; Costas *et al.*, 2004).

Amino-acid sequence comparison of ECDOs shows that they can be classified into three classes: classes I, II and III (Spence *et al.*, 1996). The amino-acid sequences of the class II enzymes suggest that they evolved from a class I enzyme through gene duplication. The class II extradiol-cleaving catecholic dioxygenases (ECDOIIs) can be further divided into two groups on the basis of the substrate specificity. Enzymes in the first group (hereafter referred to as the XylE group) have a similar substrate specificity to XylE (Nakai *et al.*, 1983; Kobayashi *et al.*, 1995). XylE shows a high substrate specificity for catechol, which is composed of one aromatic ring. Enzymes in the second group (hereafter referred to as the BphC group) have a similar substrate specificity to BphC (Kimbara *et al.*, 1989). BphC shows a high substrate specificity for 2,3-dihydroxybiphenyl, which has two aromatic rings. Because the ECDOIIs are the key enzymes of aromatic compound degradation in bacteria, the mechanisms of their catalytic reaction and their substrate specificity have attracted the attention of many researchers. There have been numerous reports regarding their catalytic mechanism (Sato *et al.*, 2002; Bugg, 2003; Viggiani *et al.*, 2004). We recently determined the crystal structures of reaction intermediates of BphC and using these results have proposed a catalytic reaction mechanism of the enzyme (Sato *et al.*, 2002). On the other hand, the mechanism of the substrate specificity is not well understood. In order to elucidate the mechanism of the substrate specificity, detailed structural comparison between the enzymes of the two groups is required. Although the crystal structures of enzymes in the BphC group have been determined at high resolution (Sugiyama *et al.*, 1995; Han *et al.*, 1995), those of the XylE-group enzymes have only been determined to 2.8 Å resolution (Kita *et al.*, 1999). A high-resolution crystal structure of an XylE-group ECDOII has not yet been obtained.

We have studied one of the XylE-group enzymes, PheB (Kim & Oriel, 1995), which is derived from *Bacillus stearothermophilus* BR219 and is a thermostable ECDOII. The enzyme is a homo-

Table 1
Diffraction data-collection statistics for PheB.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters (Å)	a = 65.5, b = 119.2, c = 158.7
Space group	$P2_12_12_1$
V_M (Å^3 Da^{-1})	2.3
Solvent content (%)	45.6
No. of subunits in the ASU	4
Resolution (Å)	50–2.3 (2.38–2.30)
Observations	350340
Unique reflections	57495
R_{merge}	0.081 (0.282)
Completeness (%)	98.7 (95.6)
$I/\sigma(I)$	13.9 (4.8)
Redundancy	6.1 (5.4)

tetramer (MW = 138.7 kDa) and shows high amino-acid sequence similarity to XylE (41%). The crystal structure of PheB has already been solved at 2.8 Å resolution by the molecular-replacement method (Sugimoto, 2000). However, we have continued our efforts to obtain a high-quality crystal of PheB because the 2.8 Å resolution crystal structure is insufficient to make a detailed structural comparison with the BphC-group enzymes. In addition, the detailed crystal structure would be useful in revealing the mechanism of the enzyme's thermostability. We have recently developed a new purification procedure for PheB and succeeded in obtaining a new crystal of PheB that diffracts to 2.3 Å resolution. Here, we present the new crystal form of PheB.

2. Methods and results

2.1. Protein expression and purification

All purification steps were carried out at 277 K under aerobic conditions. *Escherichia coli* (JM109) cells were transformed with an expression plasmid containing the *pheb* gene. Cells were grown in Luria broth medium. Expression of PheB was induced by IPTG when the culture reached an OD_{600} of 0.6–1.0. After 3 h induction, the cells were harvested, washed twice with 50 m*M* potassium phosphate buffer pH 7.5 and resuspended in the same buffer. The cells were then disrupted by sonication using a 500 W ultrasonic disintegrator (VCX500, SONICS). Cell debris and larger particles were removed by centrifugation at 9800*g* for 12 h.

The supernatant solution was fractionated by the addition of pulverized ammonium sulfate to 30%(*w*/*v*). After centrifugation at 9800*g* for 1 h, the precipitate was discarded. The obtained supernatant fraction, the pH of which was adjusted to 7.5, was applied onto a hydrophobic interaction column (Phenyl Sepharose High Performance, Amersham Biotech, 2.6 × 20 cm) which was pre-equilibrated with 30%(*w*/*v*) ammonium sulfate and 0.1 *M* potassium phosphate buffer pH 7.5. The protein was eluted with a linear ammonium sulfate gradient [30–0%(*w*/*v*)]. The fractions containing enzyme activity were combined and dialyzed against 50 m*M* potassium phosphate buffer pH 7.5. The dialyzed protein solution was applied onto a DEAE column (DEAE-Toyopearl, Tosoh, 2.2 × 20 cm) which was pre-equilibrated with 50 m*M* potassium phosphate buffer pH 7.5. The proteins were eluted with a linear NaCl gradient (80–400 m*M*). The obtained PheB fractions were combined and concentrated to 40 mg ml^{-1} by ultrafiltration using Centricon YM-10 devices (Millipore). During the ultrafiltration, the buffer was exchanged with Milli-Q water. The purified enzyme was active with a specific activity of approximately 0.4 U mg^{-1}. Incubation of PheB with Fe^{2+} and ascorbate increased its activity by approximately sevenfold. The obtained PheB enzyme was of near-homogeneity and was used for crystallization.

2.2. Crystallization

Crystallization of PheB was performed by the hanging-drop vapour-diffusion method. A hanging drop was prepared by mixing equal volumes (1 µl each) of protein solution and reservoir solution. Each hanging drop was placed over 1 ml reservoir solution. Although we obtained a crystal of PheB (Sugimoto *et al.*, 1999), we carried out a sparse-matrix screen using Crystal Screen I (Hampton Research) in order to obtain high-quality crystals. The sparse-matrix screen gave small crystals with solution Nos. 12, 17 and 38. These conditions were optimized by changing the pH, precipitant concentration, temperature and protein concentration. Although the quality of the crystals did not improve using condition Nos. 12 and 38, a high-quality crystal was obtained through the optimization of condition No. 17. The best crystals were obtained after 7 d at 285 K using 0.2 *M* Li_2SO_4, 15% PEG 4000 and 0.1 *M* HEPES pH 7.0 in the reservoir and protein solution with a concentration of 40 mg ml^{-1}. The obtained crystal was rod-shaped, with typical dimensions of 0.6 × 0.14 × 0.07 mm (Fig. 1).

2.3. Data collection

Data collection was performed at NW12 of PF-AR (Tsukuba, Japan). The PheB crystal was soaked in cryoprotectant solution [22.5%(*w*/*v*) PEG 4000, 0.3 *M* $LiSO_4$, 0.1 *M* HEPES pH 7.0 and saturated trehalose] for 30 s, mounted on a cryoloop (Hampton Research) and frozen in an N_2 stream at 100 K. Data collection was carried out using an ADSC Quantum 210 CCD camera. The collected data were processed and scaled using the programs *DENZO* and *SCALEPACK* as implemented in the *HKL*2000 package (Otwinowski & Minor, 1997). The space group of the crystal was found to be $P2_12_12_1$, with unit-cell parameters a = 65.5, b = 119.2, c = 158.7 Å. Assuming the presence of one tetramer in the asymmetric unit, a V_M value of 2.3 Å^3 Da^{-1} and a corresponding solvent content of 45.6% were obtained. Data-collection statistics are shown in Table 1.

Crystal structure analysis was carried out by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The subunit structure of the previously determined structure of PheB (Sugimoto, 2000) was used as a search model. Four subunits in the asymmetric unit were successfully determined and were found to be related to one another by 222 point-group symmetry. Packing analysis showed that there was

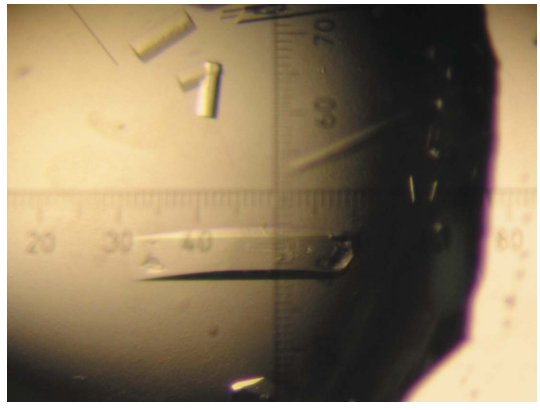

Figure 1
Crystals of PheB. The largest dimension of the crystal is approximately 0.6 mm.

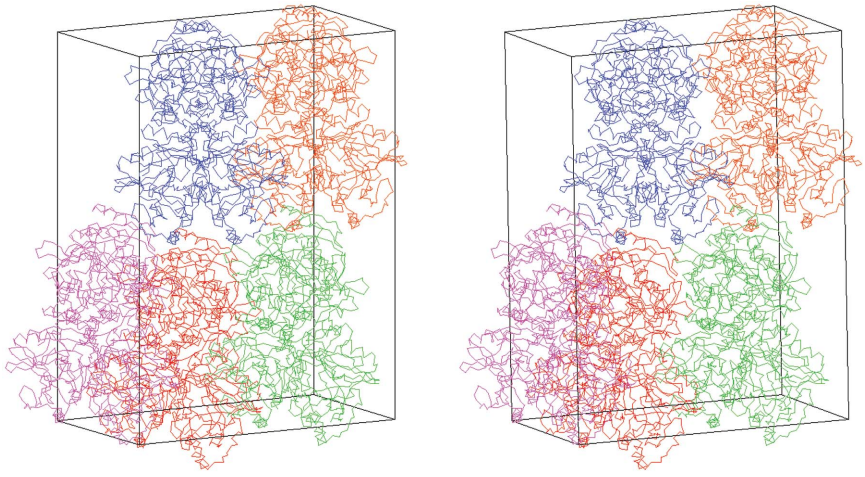

Figure 2
Packing of tetrameric PheB molecules in their orthorhombic unit cell (stereoview). This figure was prepared using the program *MolViewX* (Smith, 2004).

no close contact between the subunits (Fig. 2). Crystallographic refinement at 2.3 Å resolution is now in progress.

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