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Crystallization and preliminary crystallographic analysis of the 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase from the carbazole-degrader *Pseudomonas resinovorans* strain CA10

Kenichi Iwata,[a] Haruko Noguchi,[a] Yusuke Usami,[a] Jeong-Won Nam,[a] Zui Fujimoto,[b] Hiroshi Mizuno,[b] Hiroshi Habe,[a] Hisakazu Yamane,[a] Toshio Omori[a]‡ and Hideaki Nojiri[a]*

[a]Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, and [b]Department of Biochemistry, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

‡ Present address: Shibaura Institute of Technology, Shibaura 3-9-14, Minato-ku, Tokyo 108-8548, Japan.

Correspondence e-mail: anojiri@mail.ecc.u-tokyo.ac.jp

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CarBaBb, the class III extradiol dioxygenase involved in carbazole degradation by *Pseudomonas resinovorans* CA10, was crystallized at 278 K by the hanging-drop vapour-diffusion method using PEG MME 550 as a precipitant. The crystals had a transparent thin square-pillar shape and belonged to space group $P2_12_12$, with unit-cell parameters a = 122.8, b = 144.6, c = 49.2 Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals diffracted to a maximum resolution of 1.9 Å and gave a data set with an overall $R_{\rm merge}$ of 5.7% and a completeness of 98.6%. The $V_{\rm M}$ value was 2.52 Å^3 Da^{-1}, which indicated a solvent content of 51.2%.

1. Introduction

Extradiol dioxygenase is one of the key enzymes in the aerobic degradation of aromatic compounds. This enzyme has a non-haem Fe^{II} at its active site and catalyzes aromatic-ring cleavage at the C—C bond adjacent to the vicinal hydroxyl groups (*meta*-cleavage). All extradiol dioxygenases can be classified into three classes: classes I–III (Spence *et al.*, 1996). Sequence comparison indicates that extradiol dioxygenases can be classified into two primary divisions: class II (or I) and class III. The class II (or I) enzymes all contain the Prosite consensus sequence, whereas the class III includes enzymes whose sequences could not be aligned with those of class I or II enzymes. While the class I members are comprised of smaller subunits (162–190 amino acids), the class II enzymes have an ∼300 amino-acid subunit. Based on alignment studies, the class II enzyme is considered to have evolved from a class I enzyme through gene duplication (Spence *et al.*, 1996; Eltis & Bolin, 1996). This hypothesis is supported by the fact that the class II enzymes have two domains with similar folds, as seen in the crystal structures of several class II extradiol dioxygenases (Han *et al.*, 1995; Senda *et al.*, 1996; Kita *et al.*, 1999; Vetting *et al.*, 2004). Of the class III extradiol dioxygenases, a three-dimensional structure has only been reported for protocatechuate 4,5-dioxygenase from *Sphingomonas paucimobilis* strain SYK-6 (LigAB; PDB code 1bou; Sugimoto *et al.*, 1999). The LigAB structure does not exhibit any similarity to those of class II enzymes. Despite the completely unrelated manner of polypeptide-chain folding, the active site of LigAB shares many common geometrical characteristics with those of the class II enzymes, implying that essentially the same reaction mechanism is adopted by the class I, II and III enzymes. Thus, comparison of class II and III enzymes provides a clear example of convergent evolution of two kinds of proteins of distinct ancestry.

2′-Aminobiphenyl-2,3-diol 1,2-dioxygenase (CarBaBb) is the second enzyme in the aerobic degradation pathway of carbazole by *Pseudomonas resinovorans* strain CA10 and catalyzes the ring cleavage of 2′-aminobiphenyl-2,3-diol (Fig. 1; Nojiri & Omori, 2002). This class III extradiol dioxygenase has an $\alpha_2\beta_2$ subunit composition with a molecular weight of about 78 kDa (Iwata *et al.*, 2003). While almost all extradiol dioxygenases have a homomultimeric composition, a small number of class III members have a heteromultimeric structure. Examples of such novel extradiol dioxygenases are LigAB and CarBaBb. Both LigAB and CarBaBb have the same subunit composition,

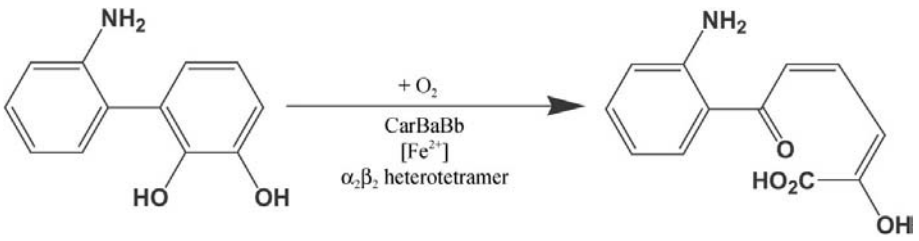

Figure 1
Enzymatic reaction catalyzed by CarBaBb.

but the homology observed between their components is rather low. From the result of an alignment using *CLUSTALW* (Thompson et al., 1994), the overall lengthwise identity of the amino-acid sequence of the large (catalytic) subunit was about 31% and there were some apparent gaps over the entire range (CarBb, 269 amino acids; LigB, 302 amino acids; Sato *et al.*, 1997). The amino-acid length of the small subunit CarBa (90 amino acids) was smaller than that of LigA (139 amino acids) and the sequence seemed to be phylogenetically unrelated (about 13% amino-acid sequence identity over the entire amino-acid range). In addition, the chemical structures of their respective primary substrates (2′-aminobiphenyl-2,3-diol for CarBaBb and protocatechuate for LigAB) differ greatly from each other. These facts suggest a possible difference in the three-dimensional structure of both enzymes. Here, we report the crystallization and preliminary X-ray diffraction studies on a novel class III extradiol dioxygenase, 2′-aminobiphenyl-2,3-diol.

2. Materials and methods

2.1. Protein expression and purification

The plasmid used for expression of the CarBaBb enzyme was constructed according to a method described previously (Iwata *et al.*, 2003), which was modified to introduce a six-histidine tag at the C-terminus of the CarBb protein. By PCR, using the *carBaBb* gene-containing plasmid pUCA122 (Sato *et al.*, 1997) as a template and an appropriate primer set, we obtained DNA fragments containing the *carBa* or modified *carBb* genes. After restriction-enzyme digestion, these two fragments were ligated in tandem. Two copies of the resultant fragment containing the *carBaBb* gene set were tandemly inserted into pUC119, forming a six-histidine-tagged CarBaBb (ht-CarBaBb) expression vector, pUCA508. *Escherichia coli* strain BL21 (DE3) was transformed by this plasmid and ht-CarBaBb protein was expressed by isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The crude cell extract of the *E. coli* cells harbouring pUCA508 showed clear *meta*-cleavage activity for 2,3-dihydroxybiphenyl, which is an analogue of the primary substrate of this enzyme, 2′-aminobiphenyl-2,3-diol. After purification by two column-chromatography steps, metal-chelating chromatography (HiTrap Chelating; Amersham Bioscience, NJ, USA) and gel-filtration chromatography (Superdex-200; Amersham Bioscience), about 50 mg ht-CarBaBb was obtained from 1 l culture. The protein was concentrated and buffer-exchanged using a Vivaspin 20 membrane (10 000 MWCO; Sartorius KK, Gottingen, Germany). Protein concentrations were estimated with a Protein assay kit (Bio-Rad; Bradford, 1976) using BSA as a standard.

2.2. Crystallization

For crystallization experiments, the concentration of ht-CarBaBb in 20 m*M* Tris–HCl pH 7.5 containing 10% glycerol was first adjusted to 10–20 mg ml^{-1}. Crystallization was performed using the hanging-drop or sitting-drop vapour-diffusion method at 278 or 293 K. Drops containing 2 µl each of protein solution and mother liquor were equilibrated against 800 µl reservoir solution. Initial crystallization screenings were performed using Crystal Screens I and II, Grid Screens (Ammonium Sulfate, PEG 600, MPD and sodium malonate) and Index kits (Hampton Research, California, USA). Thin plate-shaped crystals were obtained with Index No. 54, containing 0.05 *M* calcium chloride and 30%(*v*/*v*) PEG MME 550 in 0.1 *M* bis-Tris pH 6.5, in hanging-drop vapour-diffusion experiments at 278 K using VDX Plates (Hampton Research). In spite of the refinement of the crystallization conditions, the crystals did not grow large enough for X-ray diffraction study.

When crystallization was performed using a higher concentration (75–100 mg ml^{-1}) of ht-CarBaBb solution using the crystallization condition above, rod-shaped crystals were occasionally formed in the aggregated protein. Unfortunately, the formation of these crystals was not reproducible even with modification of the buffer conditions. However, by removing the aggregate just after mixing the protein solution with the mother liquor, we finally obtained the best crystals. In detail, a higher concentration of ht-CarBaBb solution (75–100 mg ml^{-1}) was mixed with an equivalent volume of diluted Index No. 54 by gentle inversion (about 10 s) in a 1.5 ml microtube (0.5 inversions per second). The concentrations of the constituents of the diluted Index No. 54 were as follows: 0.09 *M* bis-Tris pH 6.5, 0.045 *M* calcium chloride, 27%(*v*/*v*) PEG MME 550, 5%(*v*/*v*) glycerol. The aggregate generated by the mixing was immediately removed by centrifugation (20 000*g*, 2 h). After filtering the resultant supernatant through a 0.45 µm pore-size membrane, the filtrate obtained was directly used for hanging-drop vapour diffusion at 278 K. After the centrifugation and subsequent filtration, the concentration of CarBaBb was 13–15 mg ml^{-1} in several repeated experiments. Crystals appeared after 3 d and grew to maximum dimensions of 0.35 × 0.1 × 0.05 mm in 10 d (Fig. 2).

2.3. Data collection

Owing to the sensitivity of the crystals to X-ray radiation, cryocooling was a necessary procedure during data collection in order to avoid the crystal decay that occurred at room temperature. The crystals were mounted in a nylon loop and flash-frozen in a nitrogen stream at 100 K. Diffraction measurements of the frozen crystals were carried out using a Quantum 210 CCD X-ray detector (ADSC, California, USA) at the NW12 Photon Factory Advanced Ring (PF-AR) at the High Energy Accelerator Research Organization, Tsukuba, Japan. A total range of 360° was covered with 0.5° oscillation and a 5 s exposure per frame. All diffraction images were indexed, integrated and scaled using the *HKL*2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

A data set was collected to 1.9 Å resolution and the data-collection and processing statistics are given in Table 1. The space group was determined to be $P2_12_12$, with unit-cell parameters a = 122.8, b = 144.6, c = 49.2 Å, $\alpha = \beta = \gamma = 90.0°$. The molecular weight of the protein had been calculated to be 78 kDa from gel-filtration chromatography and the monomers of CarBa and CarBb to be approximately 10 and 29 kDa, respectively, from SDS–PAGE (Iwata *et al.*,

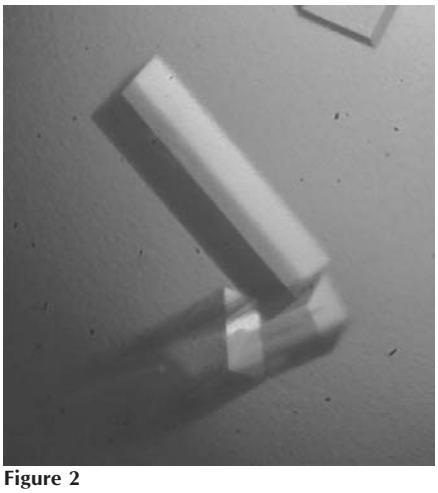

Figure 2
Photograph of taransparent square-pillar-shaped crystal of CarBaBb of *P. resinovorans* strain CA10. The crystal was grown in 0.045 *M* calcium chloride, 27%(*v*/*v*) PEG MME 550, 0.09 *M* bis-Tris pH 6.5 and 5%(*v*/*v*) glycerol. The dimensions of the crystal are approximately 0.35 × 0.1 × 0.05 mm.

Table 1
Crystal parameters and data-collection statistics.

The data set was collected on NW12 at the PF-AR, Tsukuba, Japan. Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12$
Unit-cell parameters (Å, °)	a = 122.8, b = 144.6, c = 49.2, $\alpha = \beta = \gamma = 90$
Wavelength (Å)	1.000
Resolution range (Å)	50–1.90 (1.97–1.90)
Total No. reflections	778047
No. unique reflections	67931 (6854)
Completeness (%)	96.6 (98.6)
$I/\sigma(I)$	10.0 (7.3)
R_{merge}	0.057 (0.229)
Redundancy	11.5 (11.3)

2003). Consequently, the protein probably has $\alpha_2\beta_2$ configuration and the asymmetric unit may contain a heterotetrameric extradiol dioxygenase molecule or two CarBa–CarBb heterodimeric molecules, suggesting a value of 2.52 Å^3 Da^{-1} for the Matthews coefficient V_M (Matthews, 1968), corresponding to a solvent content of 51.2%.

Attempting to solve the structure by the molecular-replacement method using the known structure of the class III extradiol dioxygenase LigAB (Sugimoto *et al.*, 1999) have given no useful results so far owing to the low sequence homology and the structural differences expected from the sequence alignment. We thus attempted to solve the structure using multiple-wavelength anomalous dispersion or multiple isomorphous replacement methods. As the CarBaBb enzyme has 21 methionine residues per pair of α and β subunits (a total of 359 amino-acid residues), application of the SeMet derivatives of CarBaBb enzymes to X-ray analysis would appear to be difficult. The preparation of heavy-atom derivatives is now in progress.

On the basis of the structure of the LigAB–protocatechuate complex, hydrogen bonds to the carboxylate O atom of the substrate and Ser269 O$^{\gamma}$ and Asn270 N$^{\delta}$ seem to be important for substrate binding (Sugimoto *et al.*, 1999). As described above, the amino-acid sequence of CarBb (catalytic subunit) shows 31% identity to that of LigB, the catalytic subunit of protocatechuate 4,5-dioxygenase, from *S. paucimobilis* strain SYK-6. However, the activity of CarBaBb toward protocatechuate was almost negligible, while 2,3-dihydroxybiphenyl and its derivatives were good substrates for *meta*-cleavage by CarBaBb (Sato *et al.*, 1997; Iwata *et al.*, 2003). The difference in the substrate specificities of the extradiol dioxygenases is thought to derive from the different three-dimensional structure of the substrate-binding pocket. In this study, we succeeded in crystallizing CarBaBb as the second example of a class III extradiol dioxygenase and comparison of the anticipated CarBaBb structure with LigAB structure will provide us with information on the molecular mechanism of substrate recognition of this novel extradiol dioxygenase. Solution of the structure will also provide a fundamental framework for understanding the structure–function relationship among class III extradiol dioxygenases.

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