Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, purification, crystallization and preliminary crystallographic studies of *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase from *Pandoraea pnomenusa* B-356

Sakshi,[a] Dipak N. Patil,[a] Shailly Tomar,[a] Michel Sylvestre[b] and Pravindra Kumar[a]*

[a]Department of Biotechnology, Indian Institute of Technology, Roorkee, Roorkee 247 667, India, and [b]Institut National de Recherche Scientifique (INRS–Institut Armand-Frappier), Pointe-Claire, Quebec H9R 1G6, Canada

Correspondence e-mail: kumarfbs@iitr.ernet.in

Received 27 August 2010
Accepted 14 September 2010

cis-Biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (BphB) is involved in the aerobic biodegradation of biphenyl and polychlorinated biphenyls. BphB from *Pandoraea pnomenusa* strain B-356 was overexpressed in *Escherichia coli*, purified to homogeneity and crystallized. Crystals were obtained by the sitting-drop vapour-diffusion method using polyethylene glycol 3350 and 0.2 *M* sodium malonate. A BphB crystal diffracted to 2.8 Å resolution and belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 75.2$, $c = 180.4$ Å. Preliminary crystallographic analysis indicated the presence of two molecules in the asymmetric unit, giving a Matthews coefficient of 2.2 Å^3 Da^{-1} and a solvent content of 44%.

1. Introduction

The ability of bacteria to grow on biphenyl and other aromatic hydrocarbons and to co-metabolically degrade polychlorinated biphenyls (PCBs) has been extensively studied (Pieper & Seeger, 2008). PCBs have been recognized as the most significant environmental pollutants (Furukawa, 1982). To date, several bacteria have been found to display a particular activity spectrum with regard to the type and extent of PCB congeners metabolized. Some strains have a narrow spectrum, while others are able to convert a broad range of congeners (Bopp, 1986; Haddock *et al.*, 1995; Mondello, 1989; Seeger *et al.*, 1995*a*,*b*, 1997, 1999). The versatile enzymes of the biphenyl-degrading pathway (encoded by the *bph* operon) can co-metabolically transform several PCBs. This pathway consists of four steps. The first-step reaction converts biphenyl to *cis*-2,3-dihydro-2,3-dihydroxybiphenyl using 2,3-biphenyl dioxygenase, which is encoded by *bphAEFG* in *Pandoraea pnomenusa* strain B-356 (Sylvestre *et al.*, 1996); *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (encoded by *bphB*) catalyzes the second step and produces 2,3-dihydroxybiphenyl. The third step in the catabolic pathway is catalyzed by a 2,3-dihydroxybiphenyl 1,2-dioxygenase (encoded by *bphC*), which cleaves the aromatic ring and produces 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, which is converted to benzoate and *cis*-2-hydroxypenta-2,4-dienoate by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (encoded by *bphD*).

BphB from *Comamonas testosteroni* strain B-356 (now known as *P. pnomenusa* strain B-356; $BphB_{B356}$) has a molecular mass of 29.4 kDa and requires NAD^+ (Vezina *et al.*, 2008). The GenBank accession number for the sequence is U57451. The crystal structure of a biphenyl dihydrodiol dehydrogenase from *Burkholderia xenovorans* sp. LB400 ($BphB_{LB400}$) established that these enzymes belong to the short-chain alcohol dehydrogenase/reductase (SDR) family (Hülsmeyer *et al.*, 1998). These enzymes are able to transform several *cis*-dihydrodiol substrates (Jouanneau & Meyer, 2006; Patel & Gibson, 1974; Raschke *et al.*, 1999; Rogers & Gibson, 1977). Although the amino-acid sequence of $BphB_{B356}$ is more than 80% identical to that of $BphB_{LB400}$, *P. pnomenusa* strain B-356 and *B. xenovorans* LB400 have attracted the attention of researchers because they have different substrate specificities. Recently, the biphenyl dioxygenase from *P. pnomenusa* strain B-356 was reported to be more potent

towards the persistent congeners 2,6-dichlorobiphenyl and 2,4-dichlorobiphenyl than the corresponding enzyme from *B. xenovorans* LB400 (Gómez-Gil *et al.*, 2007). Three-dimensional structures of these enzymes from PCB-degrading bacteria from different species will help in understanding the mechanism that determines the substrate specificity clearly; therefore, it is necessary to crystallize PCB-degrading enzymes from diverse species.

In this study, we have successfully purified and crystallized the *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase from *P. pnomenusa* strain B-356. The molecular weight of the purified enzyme is about 29 400 Da. Here, we report the expression, purification, crystallization and preliminary X-ray diffraction analysis of $BphB_{B356}$.

2. Material and methods

2.1. $BphB_{B356}$ expression and purification

$BphB_{B356}$ was produced using *Escherichia coli* BL21 (DE3) harbouring plasmid pET14b expressing recombinant His-tagged BphB of *P. pnomenusa* strain B-356. The amino-acid sequence of the affinity tag present at the N-terminus of the recombinant protein is MGSSHHHHHHSSGLVPRGSHMLEDP. 1 l Luria–Bertani broth (LB) containing 80 µg ml^{-1} ampicillin was inoculated with a 10 ml overnight culture of *E. coli* BL21 (DE3) cells containing the expression plasmid for $BphB_{B356}$ and allowed to grow at 310 K. When the optical density (OD) at 600 nm reached 0.9–1.0, the temperature was reduced to 291 K and the culture was induced with 0.5 m*M* IPTG. Induction was allowed to proceed for ∼16 h at 291 K before harvesting the cells by centrifugation (8000*g*, 10 min, 279 K).

For purification, the cell pellet was washed two times with purification buffer (PB buffer) comprised of 20 m*M* HEPES pH 8.0, 10% glycerol, 300 m*M* NaCl and resuspended in 40 ml PB buffer containing 35 µl DNase I (1 U µl^{-1}). Cells were disrupted at 138 MPa using a one-shot cell disrupter (Constant Systems Ltd, Daventry, England). The lysate was clarified by centrifugation at 14 000*g* for 45 min at 277 K and the supernatant was collected. The supernatant was added to 3 ml Ni–NTA beads (Qiagen, USA) pre-equilibrated with PB buffer containing 5 m*M* imidazole and gently agitated at 277 K for 1 h. The supernatant/bead mixture was loaded onto a gravity-flow column and the flowthrough was collected. Column washing was subsequently performed with five column volumes of washing buffers WB1, WB2 and WB3 (20 m*M* HEPES pH 6.8, 10% glycerol, 300 m*M* NaCl and 20, 40 or 60 m*M* imidazole, respectively). The bound protein was eluted with 50 ml elution buffer (20 m*M* HEPES pH 6.8, 10% glycerol, 300 m*M* NaCl and 250 m*M* imidazole) and collected in 5 ml fractions. The collected fractions were analyzed by SDS–PAGE and those showing a single band corresponding to BphB were pooled and dialyzed against 2 l dialysis buffer (20 m*M* HEPES pH 8.0, 10% glycerol, 50 m*M* NaCl) at 277 K two times for 6 h each to remove imidazole. Purified tagged protein was incubated with thrombin (thrombin:protein ratio = 1:10) for 15 h at 277 K for proteolytic cleavage of the His tag. 1 m*M* phenylmethylsulfonyl fluoride (PMSF) was then added to stop the proteolysis reaction and the sample was analyzed by SDS–PAGE to confirm His-tag cleavage. To remove uncleaved tagged protein and the cleaved His tags, the sample was incubated with Ni–NTA beads at 277 K for 1 h. The mixture of sample and beads was loaded onto a gravity-flow column and the flowthrough containing untagged $BphB_{B356}$ was collected. Finally, to remove thrombin, the protein sample was concentrated using a 10 kDa cutoff Amicon Ultra-15 concentrator (Millipore, Bedford, Massachusetts, USA) and loaded onto a HiLoad Superdex 200 16/60 column (GE Healthcare) using an ÄKTA purifier system (GE Healthcare). The gel-filtration column was operated on an ÄKTA purifier system at 279 K with a flow rate of 0.5 ml min^{-1} and was pre-equilibrated with PB buffer. The eluate was collected in 0.5 ml fractions and the protein purity was analyzed by Coomassie Blue-stained SDS–PAGE. The purified protein was concentrated using an Amicon Ultra-4 concentrator (Millipore, Bedford, Massachusetts, USA) to ∼10 mg ml^{-1} for crystallization. Protein concentration was determined with the Bio-Rad protein-assay kit (Bio-Rad Laboratories, Hercules, California, USA) using bovine serum albumin (BSA) as a standard and the yield of purified $BphB_{B356}$ was subsequently estimated.

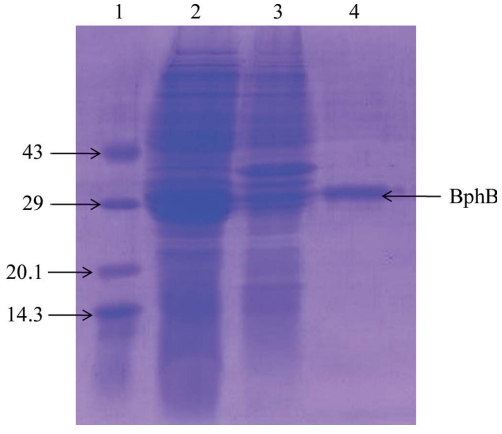

Figure 1
15% SDS–PAGE of BphB, the second enzyme of the *bph* pathway, from *P. pnomenusa* strain B-356. Lane 1, molecular-weight markers (kDa); lane 2, supernatant containing soluble protein fraction; lane 3, pellet containing insoluble protein fraction; lane 4, purified $BphB_{B356}$ protein.

2.2. Crystallization

For crystallization, purified BphB preparation was used at a concentration of ∼10 mg ml^{-1} in 20 m*M* HEPES pH 8.0, 10% glycerol and 300 m*M* NaCl. Crystallization was performed by the vapour-diffusion method in 96-well sitting-drop plates (Hampton Research, USA) at 293 K. The drops were prepared by mixing 1 µl protein solution with the same volume of reservoir solution and were equilibrated against 50 µl reservoir solution. Initial crystallization conditions were obtained by the sitting-drop vapour-diffusion method using PEG/Ion and PEG/Ion 2 reagent kits (Hampton Research, USA). Crystals of $BphB_{B356}$ were obtained using 0.2 *M* sodium malonate pH 6.0 and 20% PEG 3350 as precipitant.

2.3. Data collection and analysis

Crystals were mounted in cryoloops (Hampton Research, USA) and 20% glycerol was used as cryoprotectant by direct immersion prior to X-ray diffraction analysis. Data were collected with a MAR 345 imaging-plate system using Cu $K\alpha$ radiation generated by a Bruker–Nonius Microstar-H rotating-anode generator operated at 45 kV and 60 mA and equipped with Helios optics. Data were collected as 90 images with a crystal-to-detector distance of 280 mm and 1° oscillation per image. The time of exposure was 15 min. The crystal diffracted to 2.8 Å resolution. The diffraction data were processed with the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

$BphB_{B356}$ protein was expressed in *E. coli* BL21 (DE3) cells and purified to homogeneity. In the first step, His-tagged BphB was purified using Ni–NTA metal-affinity chromatography. The His tag was removed by proteolytic cleavage using thrombin. In the second step, the protein sample was again loaded onto an Ni–NTA column to remove uncleaved protein and the flowthrough containing His-tag-cleaved protein was collected and concentrated. In the last purification step, size-exclusion chromatography was used to remove thrombin. Gel-filtration fractions containing BphB were pooled, concentrated and assessed for homogeneity by 15% SDS–PAGE, which showed a single band of ∼29.4 kDa corresponding to the molecular weight of $BphB_{B356}$ (Fig. 1). The estimated yield was ∼10 mg of purified protein per litre of culture. Purified protein was concentrated to ∼10 mg ml^{-1} and used for crystallization.

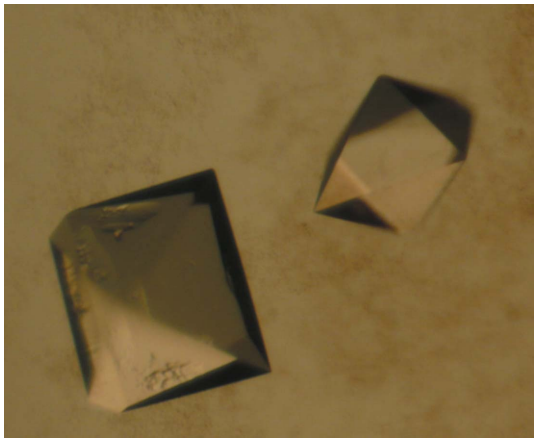

Figure 2
Crystals of $BphB_{B356}$ from *P. pnomenusa* strain B-356. The longest dimension of a typical crystal is ∼200 µm.

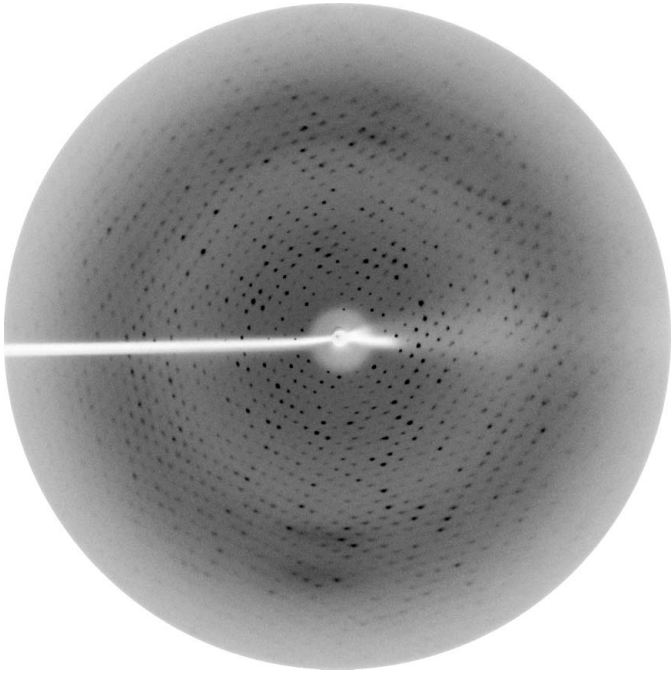

Figure 3
Diffraction of $BphB_{B356}$ crystals using in-house radiation at the Macromolecular Crystallographic Facility (MCU), IIC. The resolution at the edge of the plate is 2.8 Å.

Table 1
Data-collection statistics for BphB.

Values in parentheses are for the last resolution shell.

Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 75.2$, $c = 180.4$
Resolution range (Å)	50–2.8 (2.85–2.80)
Completeness (%)	94.3 (78.9)
R_{merge}† (%)	8.8 (44.3)
Mean $I/\sigma(I)$	6.5 (2.1)
No. of observed reflections	42464
No. of unique reflections	12223 (516)
Molecules per asymmetric unit	2
Matthews coefficient ($Å^3$ Da^{-1})	2.2
Solvent content (%)	44.0
Multiplicity	3.5 (2.4)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

The initial crystallization conditions for the $BphB_{B356}$ protein were determined by the sparse-matrix screening method (Jancarik & Kim, 1991). Diffraction-quality crystals were grown by the sitting-drop vapour-diffusion method in 3–4 d using 200 m*M* sodium malonate pH 6.0 and 20%(*w*/*v*) PEG 3350 (Fig. 2). The crystals belonged to the tetragonal space group $P4_32_12$ with two molecules in the asymmetric unit. A diffraction data set was obtained to 2.8 Å resolution, with 94.3% completeness and an R_{merge} of 8.8% (Fig. 3). Data-collection statistics are summarized in Table 1.

The *AMoRe* program package (Navaza, 1994) was used for preliminary molecular-replacement calculations. The crystal structure of tetrameric BphB from *B. xenovorans* strain LB400 (PDB code 1bdb; Hülsmeyer *et al.*, 1998), which has 80% sequence identity, was used as the search model. The best molecular-replacement solution was obtained in space group $P4_32_12$, with a correlation coefficient of 64.2 and an *R* factor of 47.5%. Currently, structure refinement is in progress. The crystal structure of BphB from *P. pnomenusa* strain B-356 will contribute to the development of rational strategies for engineering dehydrogenase enzymes in order to enhance the PCB-degrading capacities of naturally occurring bacterial strains.

The authors are grateful to and thank the Macromolecular Crystallographic Facility (MCU) at IIC, IIT Roorkee for data collection. PK and ST thank the Council of Scientific and Industrial Research (CSIR), Government of India for funding. DNP thanks MHRD, Government of India for financial support.

References

Bopp, L. (1986). *J. Ind. Microbiol.* **1**, 23–29.
Furukawa, K. (1982). *Biodegradation and Detoxification of Environmental Pollutants*, edited by A. M. Chakrabarty, pp. 33–57. Boca Raton: CRC Press.
Gómez-Gil, L., Kumar, P., Barriault, D., Bolin, J. T., Sylvestre, M. & Eltis, L. D. (2007). *J. Bacteriol.* **189**, 5705–5715.
Haddock, J. D., Horton, J. R. & Gibson, D. T. (1995). *J. Bacteriol.* **177**, 20–26.
Hülsmeyer, M., Hecht, H.-J., Niefind, K., Hofer, B., Eltis, L. D., Timmis, K. N. & Schomburg, D. (1998). *Protein Sci.* **7**, 1286–1293.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jouanneau, Y. & Meyer, C. (2006). *Appl. Environ. Microbiol.* **72**, 4726–4734.
Mondello, F. J. (1989). *J. Bacteriol.* **171**, 1725–1732.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Patel, T. R. & Gibson, D. T. (1974). *J. Bacteriol.* **119**, 879–888.
Pieper, D. H. & Seeger, M. (2008). *J. Mol. Microbiol. Biotechnol.* **15**, 121–138.
Raschke, H., Fleischmann, T., van der Meer, J. R. & Kohler, H. P. E. (1999). *Appl. Environ. Microbiol.* **65**, 5242–5246.

Rogers, J. E. & Gibson, D. T. (1977). *J. Bacteriol.* **130**, 1117–1124.
Seeger, M., Timmis, K. N. & Hofer, B. (1995*a*). *Appl. Environ. Microbiol.* **61**, 2654–2658.
Seeger, M., Timmis, K. N. & Hofer, B. (1995*b*). *FEMS Microbiol. Lett.* **133**, 259–264.
Seeger, M., Timmis, K. N. & Hofer, B. (1997). *Marine Chem.* **58**, 327–333.
Seeger, M., Zielinski, M., Timmis, K. N. & Hofer, B. (1999). *Appl. Environ. Microbiol.* **65**, 3614–3621.
Sylvestre, M., Sirois, M., Hurtubise, Y., Bergeron, J., Ahmad, D., Shareck, F., Barriault, D., Guillemette, I. & Juteau, J. M. (1996). *Gene*, **174**, 195–202.
Vezina, J., Barriault, D. & Sylvestre, M. (2008). *J. Mol. Microbiol. Biotechnol.* **15**, 139–151.