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Expression, purification, crystallization and initial crystallographic characterization of the *p*-hydroxybenzoate hydroxylase from *Corynebacterium glutamicum*

p-Hydroxybenzoate hydroxylase (PHBH) is an FAD-dependent monooxygenase that catalyzes the hydroxylation of *p*-hydroxybenzoate (pOHB) to 3,4-dihydroxybenzoate in an NADPH-dependent reaction and plays an important role in the biodegradation of aromatic compounds. PHBH from *Corynebacterium glutamicum* was crystallized using the hanging-drop vapour-diffusion method in the presence of NaH_2PO_4 and K_2HPO_4 as precipitants. X-ray diffraction data were collected to a maximum resolution of 2.5 Å on a synchrotron beamline. The crystal belongs to the hexagonal space group $P6_322$, with unit-cell parameters $a = b = 94.72$, $c = 359.68$ Å, $\gamma = 120^\circ$. The asymmetric unit contains two molecules, corresponding to a packing density of 2.65 Å^3 Da^{-1}. The structure was solved by molecular replacement. Structure refinement is in progress.

1. Introduction

p-Hydroxybenzoate hydroxylase (PHBH) plays an important role in the biodegradation of aromatic compounds and is used for decontamination procedures such as textile, paper and pulp processing, and water treatment. *Pseudomonas aeruginosa* and *P. fluorescens* have been used extensively as a model for the reactions catalyzed by flavoprotein monooxygenases, particularly for enzymes that oxygenate aromatic compounds (Entsch *et al.*, 2005). PHBH is a homodimer with a monomer molecular weight of 45 kDa; it contains one FAD per molecule and catalyzes the oxygenation of *p*-hydroxybenzoate (pOHB) to 3,4-dihydroxybenzoate (Entsch & van Berkel, 1995; Fig. 1). The catalytic cycle is divided into two half-reactions: the

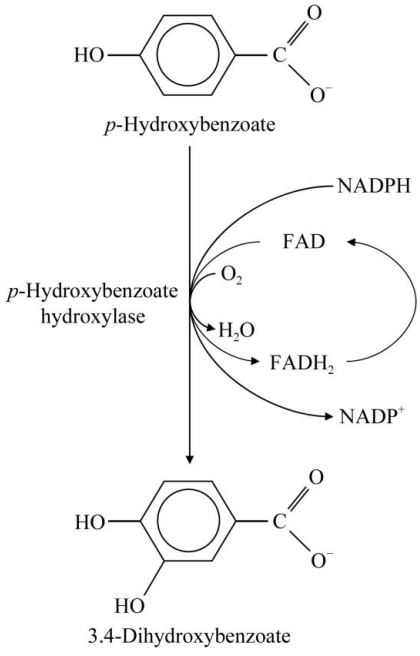
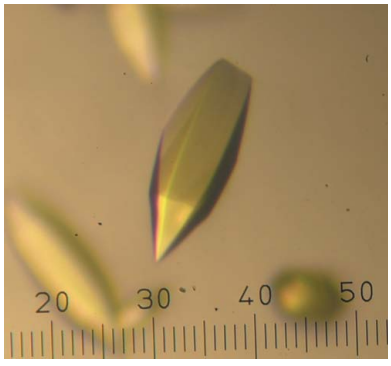


Figure 1
Enzyme reaction catalyzed by *p*-hydroxybenzoate hydroxylase. The reaction involves FAD-dependent oxygenation of the aromatic compound coupled to oxidation of NADPH.



reductive half-reaction, in which the flavin prosthetic group is reduced by NADPH, and the oxidative half-reaction, in which the flavin reacts with molecular oxygen to from a flavin C(4a)-hydroperoxide that hydroxylates the bound substrate (Husain & Massey, 1979). In PHBH, the isoalloxazine of the FAD moves between two different positions to carry out two different reactions at sites that are near to each other on a single polypeptide. At least three distinct conformational states play important roles in substrate binding and product release, reaction and interaction with NADPH and the reactions with molecular oxygen (Entsch *et al.*, 2005).

In addition to Gram-negative bacteria such as *Pseudomonas* species, it has been reported that some Gram-positive bacteria, including members of the genera *Corynebacterium*, *Rhodococcus* and *Streptomyces*, are able to mineralize a wide array of aromatic compounds (Eulberg *et al.*, 1998; Golovleva *et al.*, 1991; Itoh *et al.*, 1996). Comparative studies indicated there to be extensive differences between the biodegradation processes in Gram-positive and Gram-negative bacteria (Fuller & Manning, 1997). However, our understanding of aromatic degradation in Gram-positive bacteria at the genetic and biochemical levels is relatively limited. *C. glutamicum* is widely used for the industrial production of amino acids and vitamins and is known to degrade several aromatic compounds, such as styrene (Itoh *et al.*, 1996), 2,4-dichlorobenzoate (Romanov & Hausinger, 1996) and 2,4,6-trinitrotoluene (Fuller & Manning, 1997). Genomic analysis and identification of the catabolic pathways for aromatic compounds in *C. glutamicum* revealed that the organism contains multiple ring-cleavage pathways for the degradation of aromatic compounds (Shen *et al.*, 2005).

Extensive crystallographic studies have been reported on PHBH from *P. fluorescens* and *P. aeruginosa* and revealed the enzyme mechanism in detail (Wierenga *et al.*, 1979; Schreuder *et al.*, 1994; Gatti *et al.*, 1996). Recently, the crystal structure of PHBH from *Comamonas testosteroni*, which has an identity and similarity to *Corynebacterium glutamicum* PHBH (CgPHBH) of 24% and 39%, respectively, was also reported (Hiromoto *et al.*, 2006). Nevertheless, no structure–function study has been reported for the biodegradation of aromatic compounds by a Gram-positive bacterium. As a step towards elucidating the detailed catalytic mechanism of a PHBH from a Gram-positive bacterium, we cloned the PHBH gene from the chromosome of *C. glutamicum* ATCC 13032, purified the protein and assessed its ability to convert *p*-hydroxybenzoate to 3,4-dihydroxybenzoate using liquid-chromatography mass spectroscopy (LCMS; unpublished data). We obtained crystals of the CgPHBH protein. The crystals diffracted well and data were collected to a resolution of 2.5 Å. Here, we describe the expression, purification, crystallization and initial crystallographic analysis of the CgPHBH protein.

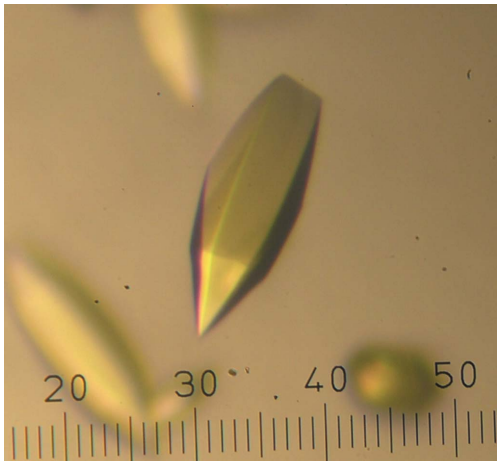


Figure 2
Hexagonal crystal of CgPHBH. The yellow colour of the crystal indicates that the protein contains FAD. The crystals have approximate dimensions of 300 × 150 × 100 µm.

2. Expression and purification of the recombinant CgPHBH protein

The gene encoding CgPHBH was amplified from *C. glutamicum* chromosomal DNA by polymerase chain reaction (PCR) using the following primers: forward primer 5′-GCGCG**CATATG**AACCACG-TACCAGTGGCAATTATTG-3′ and reverse primer 5′-GCGCG**CT-CGAG**TTATACCTCGAAGCGTGGTAGGTC-3′. The forward and reverse primers were designed to contain *Nde*I and *Xho*I restriction-enzyme sites, respectively (shown in bold). The resulting PCR product was cloned into the pPosKJ expression vector (Kwon *et al.*, 2005), which resulted in the expression of CgPHBH fused to a hexahistidine tag and bacterial haemoglobin (6×His-VHb) at its N-terminus. *Escherichia coli* B834 cells transformed with the resulting plasmid, termed pPosKJ:*Cgphbh*, were then used to produce 6×His-VHb-fused CgPHBH protein as follows. A 20 ml overnight culture was inoculated into 1 l Luria–Bertani (LB) medium supplemented with ampicillin, which was then incubated at 310 K. When the culture reached log phase (OD_{600} = 0.6), expression of the 6×His-VHb-fused protein was induced by adding IPTG to a final concentration of 1 m*M*. The cells were grown for an additional 16 h at 295 K. The harvested cells, which were red, were resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 5 m*M* *β*-mercaptoethanol) and disrupted by ultrasonication.

The crude cell extracts were centrifuged at 11 000*g* for 1 h at 277 K. The target proteins from the clarified cell lysates were loaded onto an Ni–NTA column (Qiagen) equilibrated with buffer *A*. After washing the column with buffer *A* containing 20 m*M* imidazole, bound proteins were eluted with buffer *A* containing 300 m*M* imidazole. The eluted fraction was loaded onto a HiTrap Q anion-exchange column (Amersham Biosciences) and the bound proteins were eluted using a 0–500 m*M* linear gradient of NaCl. The 6×His-VHb was removed by incubation with recombinant tobacco etch virus (rTEV) protease (Invitrogen) and passage through an Ni–NTA column. After purification, CgPHBH still had a two-residue cloning artifact (Gly-His) at its N-terminus. The final yield of the protein was approximately 45 mg per litre of cell culture. The purified protein was dialyzed against 20 m*M* Tris–HCl pH 7.0 containing 5 m*M* *β*-mercaptoethanol, concentrated to 15 mg ml^{-1} and stored at 193 K for use in crystallization trials. The molecular weight of the purified protein was analyzed by size-exclusion chromatography. The purified CgPHBH protein was loaded onto a Superdex 200 column equilibrated with 50 m*M* Tris pH 8.0 and 150 m*M* NaCl. Size-exclusion chromatography showed the molecular weight of CgPHBH to be approximately 90 kDa (data not shown), whereas SDS–PAGE suggested a molecular weight of approximately 44.0 kDa, indicating that CgPHBH probably exists as a homodimer under physiological conditions.

3. Crystallization

Crystallization of the purified protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald Biostructures using the hanging-drop vapour-diffusion technique at 295 K. Each experiment consisted of mixing

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Beamline	4A (HFMX), PAL
Wavelength (Å)	1.0000
Temperature (K)	100
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1.0
Total rotation range (°)	180
Space group	$P6_322$
Unit-cell parameters (Å, °)	$a = b = 94.72$, $c = 359.68$, $\gamma = 120$
Resolution limits (Å)	50.0–2.5 (2.59–2.5)
Total reflections	472877
Unique reflections	34389
Completeness (%)	96.0 (72.8)
Wilson B factor (Å^2)	60.3
$R_{\rm merge}$† (%)	5.3 (24.4)
$I/\sigma(I)$	44.8 (3.26)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i - I_{\rm m}|/\sum_{hkl}\sum_i I_i$, where I_i and $I_{\rm m}$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections.

2 µl protein solution (15 mg ml^{-1} in 20 mM Tris–HCl pH 7.0 and 5 mM β-mercaptoethanol) with 2 µl reservoir solution and then equilibrating the drop against reservoir solution. CgPHBH crystals were observed from several crystallization screening conditions. After several steps that improved the crystallization process, the best quality crystals appeared in 2–3 d and reached maximal dimensions of approximately 300 × 150 × 100 µm using reservoir solution containing 0.75 M NaH_2PO_4, 1.13 M K_2HPO_4, 3.3% glucose and 0.1 M acetate pH 4.5 (Fig. 2). The yellow colour of the crystals indicates that the protein contains FAD as a cofactor.

4. X-ray analysis

The crystals were transferred to cryoprotectant solution containing 0.75 M NaH_2PO_4, 1.13 M K_2HPO_4, 3.3% glucose, 0.1 M acetate pH 4.5 and 20% glycerol, fished out with a loop larger than the crystals and flash-frozen by immersion in liquid nitrogen at 100 K. Data were collected to a resolution of 2.5 Å at beamline 4A (HFMX) of the Pohang Accelerator Laboratory (PAL, Pohang, Korea) using a Quantum 210 CCD detector (ADSC, USA). A total of 180 images were collected with an oscillation angle of 1° and an exposure time of 5 s per image. The data were indexed, integrated and scaled using the *HKL*-2000 package (Otwinowski & Minor, 1997). The CgPHBH crystals belong to the hexagonal space group $P6_322$, with unit-cell parameters $a = b = 94.72$, $c = 359.68$ Å, $\gamma = 120°$. Assuming two molecules of CgPHBH per asymmetric unit, the crystal volume per unit of protein weight was 2.65 Å^3 Da^{-1} (Matthews, 1968), which corresponds to a solvent content of approximately 53.51%. Crystallographic data statistics are summarized in Table 1.

We have attempted molecular-replacement methods of phase determination and a solution was found using the *P. aeruginosa* PHBH protein (PDB code 1iuu) with side chains converted to Ala as a search model. The identity and similarity of the 1iuu sequence to that of CgPHBH are 39% and 57%, respectively. *MOLREP* (Vagin & Teplyakov, 1997) located two polyalanine-model molecules in the asymmetric unit. The resulting solution had a correlation coefficient and R factor of 0.432% and 60.2%, respectively. After rigid-body refinement using *REFMAC*5 (Murshudov *et al.*, 1997) from the *CCP*4 suite in the resolution range 50–2.5 Å, R and $R_{\rm free}$ were 54.7% and 55.6%, respectively, with a correlation coefficient of 0.642. The initial electron-density map, which was of good quality with backbones well defined by electron density, allowed us to build a three-dimensional CgPHBH model. Crystallographic model building and refinement of the structure to 2.5 Å resolution are in progress.

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