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Structural comparison of *p*-hydroxybenzoate hydroxylase (PobA) from *Pseudomonas putida* with PobA from other *Pseudomonas* spp. and other monooxygenases

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The crystal structure is reported of *p*-hydroxybenzoate hydroxylase (PobA) from *Pseudomonas putida*, a possible drug target to combat tetracycline resistance, in complex with flavin adenine dinucleotide (FAD). The structure was refined at 2.2 Å resolution with four polypeptide chains in the asymmetric unit. Based on the results of pairwise structure alignments, PobA from *P. putida* is structurally very similar to PobA from *P. fluorescens* and from *P. aeruginosa*. Key residues in the FAD-binding and substrate-binding sites of PobA are highly conserved spatially across the proteins from all three species. Additionally, the structure was compared with two enzymes from the broader class of oxygenases: 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *P. nitroreducens* and 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (MHPCO) from *Mesorhizobium japonicum*. Despite having only 14% similarity in their primary sequences, pairwise structure alignments of PobA from *P. putida* with HbpA from *P. nitroreducens* and MHPCO from *M. japonicum* revealed local similarities between these structures. Key secondary-structure elements important for catalysis, such as the $\beta\alpha\beta$ fold, β -sheet wall and α 12 helix, are conserved across this expanded class of oxygenases.

1. Introduction

Antibiotic resistance has become a growing concern, as there are over two million reported infections and 23 000 deaths annually in the United States alone (Centers for Disease Control and Prevention, 2018). The Gram-negative bacterium *Pseudomonas putida*, which is normally a soil microbe, has emerged as a bacterial species responsible for hospital-acquired infections in France (Loiseau-Marolleau & Malarre, 1977), Italy (Docquier *et al.*, 2003) and Japan (Yomoda *et al.*, 2003). The majority of bacterial infections in humans are related to the use of drainage tubes and catheters (Yoshino *et al.*, 2011). Of concern, some *P. putida* isolates have acquired resistance to quinolones, aminoglycosides, β -lactams and polyketides, among various other antibiotics (Molina *et al.*, 2014). These strains of *P. putida* harbor plasmids that can transfer antibiotic resistance to the more prevalent pathogen *P. aeruginosa* via horizontal gene transfer (Yomoda *et al.*, 2003).

The metabolism of *P. putida* is highly versatile, as it has evolved to adapt to different environments (Tang *et al.*, 2011). An important aspect of *P. putida* metabolism is the ability to catabolize aromatic compounds. The β -ketoadipate pathway is

the central aromatic catabolic pathway in *P. putida* and consists of two branches, the protocatechuate (PCA) and catechol pathways, that converge at β -ketoadipate enol-lactone (Jiménez *et al.*, 2002). The gene products of the *pcaDIJF* operon then catabolize β -ketoadipate enol-lactone into acetyl-coenzyme A (CoA) and succinyl-CoA (Harwood & Parales, 1996), which are directed into central carbon metabolism. In the upstream PCA branch, phenolic compounds are catabolized into β -ketoadipate. An essential step in the PCA pathway is the oxygenation of 4-hydroxybenzoate (4-HB) to 3,4-dihydroxybenzoate (also known as protocatechuate), a reaction that is catalyzed by the enzyme *p*-hydroxybenzoate hydroxylase (pHBH; Huijbers *et al.*, 2014).

pHBH, also known as PobA, is a flavin-dependent monooxygenase that utilizes one O atom from O₂ to hydroxylate 4-hydroxybenzoate, while reducing the other O atom to water (Entsch & van Berkel, 1995). The pHBH enzyme is typically a dimer consisting of two subunits, each of about 43 kDa (Wierenga *et al.*, 1979). Both subunits contain flavin adenine dinucleotide (FAD), which interacts with nicotinamide adenine dinucleotide phosphate (NADPH) and 4-HB to form a ternary complex that allows the oxygenation of 4-HB by the reduction of FAD (Entsch & van Berkel, 1995). It has been noted that benzoate, a metabolite from the alternative β -ketoadipate branch (the catechol pathway), can repress the transcription of pHBH (Donoso *et al.*, 2011). There are several well characterized crystal structures of pHBH from *P. fluorescens* and *P. aeruginosa* (Eppink *et al.*, 1999; Gatti *et al.*, 1996; Schreuder *et al.*, 1988). Work with the pHBH from *P. putida* has been highly difficult owing to the instability of the purified protein (Husain *et al.*, 1978). Here, we present the first three-dimensional crystal structure of PobA from *P. putida* in complex with FAD. The detailed study of this enzyme in antimicrobial-resistant pathogens is of interest as it is the representative member of the FAD-dependent aromatic hydrolases, which include the TetX monooxygenase associated with resistance to tetracycline.

2. Materials and methods

2.1. Protein expression and purification

The wild-type *pobA* gene (UniProtKB ID Q88H28) from *P. putida* strain KT2440 was cloned into the ampicillin-resistant pMCSG53 vector, which contains an N-terminal polyhistidine tag followed by the Tobacco etch virus (TEV) protease cleavage site and the start codon of the *pobA* gene. It was transformed into kanamycin-resistant *Escherichia coli* BL21 (DE3) Magic competent cells as described previously (Kwon & Peterson, 2014) with modifications. The cells were grown at 37°C and 220 rev min⁻¹ in 3 l Terrific Broth until the OD_{600 nm} reached 0.2 and were placed at 4°C overnight. Growth at 37°C was resumed the next morning and protein expression was induced with 0.6 mM IPTG when an OD_{600 nm} of 1.6 was reached. The protein was expressed overnight with the shaking reduced to 200 rev min⁻¹ and the temperature reduced to 22°C. The cells were harvested by centrifugation as

Table 1
Crystallization.

Method	Vapor diffusion, sitting drop
Plate type	96-well microplate
Temperature (°C)	19
Protein concentration (mg ml ⁻¹)	13.8
Protein solution	100 mM Tris–HCl pH 8.3, 5 mM BME, 1 mM FAD
Reservoir solution	200 mM potassium thiocyanate, 100 mM bis-Tris propane pH 6.5, 20% (w/v) PEG 3350
Ratio of drop	1:1
Volume of reservoir (μl)	100

described previously (Kuhn *et al.*, 2013). The resulting cell pellet was resuspended in 120 ml lysis buffer [10 mM Tris–HCl pH 8.3, 500 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine, 10% (v/v) glycerol, 0.01% (v/v) IGEPAL CA-630, EDTA-free protease inhibitors (Roche; one tablet per 100 ml of buffer)] and the suspension was frozen at –20°C until purification. The frozen suspension was thawed under cold running water, sonicated and centrifuged. The protein was purified in two steps using nickel(II)-affinity chromatography followed by size-exclusion chromatography as described by Shuvalova (2014) with modification. The concentration of NaCl in the wash buffer was increased to 1.0 M in order to reduce nonspecific binding. The polyhistidine tag was cleaved by incubation of the tagged protein with recombinant TEV protease overnight at 20°C. The purity of the protein was assessed by SDS–PAGE and its oligomeric state was estimated by size-exclusion chromatography (SEC) as well as native gel electrophoresis. SEC was performed on a Superdex 200 10/300 GL column using 10 mM Tris–HCl pH 7.5, 150 mM NaCl after calibration using the Gel Filtration Calibration Kit (GE Healthcare Life Sciences). For native gel electrophoresis, the protein was diluted to 4, 6 and 8 mg ml⁻¹ in loading solution consisting of 50 mM NaCl, 50 mM imidazole–HCl pH 7.0, 2 mM aminocaproic acid (Wittig *et al.*, 2007). Samples (1 μl) and commercial standards (NativeMark, Life Technologies) were applied onto the gel (10–15% gradient) and separated by electrophoresis (Phast System, GE Healthcare). The gel was fixed in solution (40% ethanol, 10% acetic acid) and stained overnight with Bio-Safe Coomassie G-250 stain (Bio-Rad). The background was washed out with deionized water.

2.2. Crystallization

For crystallization screening, the pure protein was dialyzed against 10 mM Tris–HCl pH 8.3 buffer and concentrated to 13.8 mg ml⁻¹ in 10 mM Tris–HCl pH 8.3, 5 mM β -mercaptoethanol (BME), 1 mM FAD in the presence or absence of 500 mM NaCl. 2 μl crystallization drops consisting of a 1:1 ratio of protein and reservoir solutions were then equilibrated against 96 conditions per screen using the commercially available The Classics II, PACT and JCSG+ Suites (Qiagen). Diffraction-quality crystals of PobA grew from 200 mM potassium thiocyanate, 100 mM bis-Tris propane pH 6.5, 20% (w/v) PEG 3350 (condition F4 from The PACT Suite) using protein solution without NaCl (Table 1).

2.3. Data collection and processing

Prior to flash-cooling in liquid nitrogen, crystals of PobA were transferred into a 5 μ l drop of reservoir solution, which served as a cryoprotectant. Data were collected on the LS-CAT 21-ID-F beamline at the Advanced Photon Source (APS) at Argonne National Laboratory. A total of 350 images, which corresponded to 210° of spindle-axis rotation, were indexed, integrated and scaled using *HKL-3000* (Minor *et al.*, 2006). Data-collection and data-processing statistics are listed in Table 2.

2.4. Structure solution and refinement

The structure of PobA in complex with FAD was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite (Winn *et al.*, 2011) using the structure of wild-type pHBH from *P. fluorescens* (PDB entry 1pbb; Schreuder *et al.*, 1994) as a search model. The initial solution underwent several rounds of refinement in *REFMAC* v.5.5 and manual model corrections using *Coot* (Emsley & Cowtan, 2004). Water molecules were generated using *ARP/wARP* (Morris *et al.*, 2003), and FAD and other ligands were added to the model

Figure 1

Three-dimensional crystal structure of PobA from *P. putida*. (a) Ribbon representation of the crystallized PobA dimer. Each monomer of the structure is represented with coloration depicting its three distinct domains: domains I, II and III are colored green, orange and blue, respectively. (b) The PobA monomer separated into its three distinct domains. Domain I (green), domain II (orange) and domain III (blue) are each annotated and separated in labeled boxes. α -Helices and β -strands are labeled.

Table 2
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 21-ID-F, APS
Wavelength (Å)	0.97872
Temperature (K)	100
Detector	MAR Mosaic 300 mm CCD
Space group	$P2_1$
a, b, c (Å)	65.04, 152.30, 88.64
α, β, γ (°)	90.00, 102.43, 90.00
Resolution range (Å)	30.00–2.20 (2.24–2.20)
No. of unique reflections	86948 (4317)
Completeness (%)	100.0 (100.0)
Multiplicity	4.5 (4.5)
$\langle I/\sigma(I) \rangle$	23.8 (1.9)
$R_{\text{r.i.m.}}^\dagger$	0.031 (0.431)
$CC_{1/2}^\ddagger$	(0.779)
Overall B factor from Wilson plot (Å ²)	44.8

[†] Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N - 1)]^{1/2}$, where N is the data multiplicity. [‡] Pearson's correlation coefficient (Karplus & Diederichs, 2012).

manually. Translation–libration–screw (TLS) groups were created by the *TLSMD* server (Painter & Merritt, 2006; <http://skuldbmcs.washington.edu/~tlsmd/>) and TLS corrections were applied during the final stages of refinement. *MolProbity* (Chen *et al.*, 2010; <http://molprobity.biochem.duke.edu/>) was used to monitor the quality of the model during refinement and for final validation of the structure. The final model and diffraction data were deposited in the Protein Data Bank (<https://www.rcsb.org/>) with PDB code 6dll. The final model consisted of four polypeptide chains. Each chain contained 395 residues of PobA and two (chains *A, C* and *D*) or three (chain *B*) residues of the purification tag. There were four FAD molecules, 11 chloride ions, one formic acid molecule and 895 water molecules in the crystal structure. Refinement statistics and the quality of the final model are summarized in Table 3.

Table 3
Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	29.24–2.20 (2.26–2.20)
Completeness (%)	99.9 (100.0)
No. of reflections, working set	80878 (5938)
No. of reflections, test set	4303 (302)
Final R_{work}	0.167 (0.304)
Final R_{free}	0.222 (0.352)
No. of non-H atoms	
Protein	12841
Ligand	235
Water	949
Total	14025
R.m.s. deviations	
Bonds (Å)	0.008
Angles (°)	1.284
Average B factors (Å ²)	
Protein	45.7
Ligand	39.8
Water	40.7
Ramachandran plot	
Favored regions (%)	98.0
Additionally allowed (%)	2.0
Outliers (%)	0.0

3. Results

3.1. Overall three-dimensional structure of PobA

The crystal structure of PobA from *P. putida* was determined to 2.2 Å resolution with four polypeptide chains in the asymmetric unit [Fig. 1(a)]. Analysis of protein interfaces in the crystal structure using *PDBEPIA* (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) suggested that PobA will form a stable homodimer in solution with 6300 Å² of buried surface area. This is supported by size-exclusion chromatography and clear native gel electrophoresis [Figs. 2(a) and 2(b)], which showed an estimated molecular weight of 85.4 kDa. Overall, PobA consists of three domains: domain I (amino acids 1–

Figure 2
(a) Size-exclusion chromatography of PobA from *P. putida*. The red curve shows the PobA profile and the black curve shows molecular-weight standards. Corresponding molecular weights are indicated on each peak. (b) Clear native gel electrophoresis; the asterisk indicates the band which corresponds to the dimer of PobA. Molecular-weight standards are labeled in kDa.

Figure 3

Multiple sequence alignment of monooxygenases. The alignment includes two additional flavoprotein oxygenases: HbpA from *P. nitroreducens* (PDB entry 4cy8) and MHPCO from *M. japonicum* (PDB entry 5hxi). Residues on a red background show complete conservation across all five enzymes. Residues outlined in blue are residues in PobA from *P. putida* that are conserved across two other enzymes. *ClustalW* (Larkin *et al.*, 2007) and *ESPrpt* (Robert & Gouet, 2014) were used to generate the multiple sequence alignment.

180), which includes the FAD-binding pocket, domain II (amino acids 181–289), which includes the 4-HB substrate-binding pocket, and domain III (amino acids 290–395), which provides structural support for the enzyme and serves as a dimerization interface between two pHBH monomers (Schreuder *et al.*, 1988). Each PobA chain in the structure

contains 20 β -strands and 15 α -helices, as denoted in Fig. 1(b). Several of these α -helices ($\alpha 2$, $\alpha 5$, $\alpha 7$, $\alpha 8$, $\alpha 10$ and $\alpha 11$) are extremely short, as has also been seen in other pHBH crystal structures (Schreuder *et al.*, 1988). There are two main conformations of PobA: an 'out' (open) state in which the isoalloxazine moiety of FAD swings out to open an aqueous

Figure 4

Structural comparison of PobA from *P. putida* with PobA from *P. fluorescens* and *P. aeruginosa*. (a) Ribbon representation of a structural alignment of PobA from *P. putida* (gray) with PobA from *P. fluorescens* (orange) and from *P. aeruginosa* (green). (b) Stick-model presentation of the FAD-binding site of PobA from *P. putida* showing the residues directly interacting with FAD. A $|2F_o - F_c|$ electron-density map contoured at the 1σ level surrounding FAD is shown as a mesh; hydrogen bonds with distances of less than 3 Å are shown as dashed lines. C, N, O and P atoms are colored gray, blue, red and orange, respectively. (c) Surface representation of the FAD- and substrate-binding sites of PobA from *P. putida* and different conformations of FAD (stick models). An 'in' conformation is observed for PobA from *P. fluorescens* (orange) and an 'out' conformation for PobA from *P. aeruginosa* (green) and *P. putida* (gray). (d) Comparison of the active-site residues of PobA from *P. putida* and *P. fluorescens*. 4-HB and residues that directly interact with 4-HB are shown as sticks, with C atoms in gray for *P. putida* and orange for *P. fluorescens*, N atoms in blue and O atoms in red. Dotted lines represent hydrogen-bond interactions between the active-site residues and the substrate.

channel for substrate flow and an 'in' (closed) state in which FAD prevents substrate flow (Gatti *et al.*, 1994).

3.2. The PobA structure is highly conserved across various organisms

For structural analysis, we compared the crystal structure of PobA from *P. putida* with the closest sequence homologs deposited in the PDB with similar resolution: (i) the 1.9 Å resolution crystal structure of *p*-hydroxybenzoate hydroxylase from *P. fluorescens* in complex with 4-oxidanylbenzoic acid and FAD (PDB entry 1pbe; Ortiz-Maldonado *et al.*, 1999) and (ii) the 2.1 Å resolution crystal structure of the pHBH from *P. aeruginosa* in complex with 2,4-dihydroxybenzoic acid and FAD (PDB entry 1dod; Gatti *et al.*, 1996). The pHBHs from *P. fluorescens* and *P. aeruginosa* share 99% sequence identity between them, but have only 75% sequence identity and 86% sequence similarity to PobA from *P. putida* (Fig. 3). The structures were also similar, with a high *Z*-scores (8.1) and low

r.m.s.d.s (~ 0.7 Å) for pairwise structure alignments of PobA from *P. putida* with *P. fluorescens* and *P. aeruginosa* pHBH using *jCE* (<http://source.rcsb.org/>). For comparison, we obtained a *Z*-score of 8.2 and an r.m.s.d. of below 0.3 Å for the pairwise structure alignment of pHBH from *P. fluorescens* and pHBH from *P. aeruginosa* using the same structure-alignment method (Prlić *et al.*, 2010). As seen in Fig. 4(a), the secondary structures are conserved and the tertiary structures are very similar for these enzymes. It should be noted that all residues that are involved in binding the cofactor and substrate are highly conserved in the pHBH structures from all three species (Fig. 3).

3.3. FAD-binding domain of PobA

As noted above, domain I of *P. putida* PobA contains the FAD-binding domain. All pHBH enzymes have an essential $\beta\alpha\beta$ fold, which is known to bind FAD (Schreuder *et al.*, 1988). In the crystal structure of PobA from *P. putida*, the $\beta\alpha\beta$ fold is

Figure 5

Superpositions of PobA from *P. putida* (gray) with (a) HbpA from *P. nitroreducens* (PDB entry 4cy8, blue) and (b) MHPCO from *M. japonicum* (PDB entry 5hxi, red).

formed by $\beta 1$, $\alpha 1$ and $\beta 2$ and has FAD bound to it [Fig. 4(b)]. The residues interacting with FAD are predominantly positively charged in order to promote binding to the negatively charged pyrophosphate moiety of FAD, as can be seen in Fig. 4(b). Ser13, Glu32, Arg42, Arg44, Gln102, Tyr222 and Asp286 all are hydrogen-bonded to FAD. Moreover, Arg44 makes a hydrophobic interaction with the flavin portion of FAD. Additionally, there are several water molecules in the structure, which form salt bridges between the protein and FAD. Examination of the FAD-binding site and the position of the isoalloxazine ring reveals that the observed structure represents an 'out' conformation [Fig. 4(c)].

3.4. Active site of PobA

It has been established that active-site residues Tyr201, Ser212, Arg214, Tyr222 and Pro293 interact with the carboxyl and phenolic components of 4-HB and are essential for its oxidative catalysis (Entsch & van Berkel, 1995). From Fig. 4(d), it is evident that not only are the amino acids in the active sites conserved, but their positions (r.m.s.d. of less than 0.4 Å) are nearly identical in both *Pseudomonas* species. Thus, it can be concluded that the active sites of pHBH are identical across various *Pseudomonas* species.

3.5. Structural similarity of PobA to other flavoprotein oxygenases

Additionally, we explored how pHBH from *P. putida* compares with other enzymes in the broader class of flavoprotein oxygenases: specifically, 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (MHPCO) from *Mesorhizobium japonicum* and 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *P. nitroreducens*. MHPCO is a flavoenzyme that catalyzes oxidative ring opening of 2-methyl-3-hydroxypyridine-5-carboxylic acid (Kobayashi *et al.*, 2017). HbpA hydroxylates its substrate 2-hydroxybiphenyl (Jensen *et al.*, 2015). The primary

sequences of MHPCO and HbpA do not closely align, with only 14% sequence identity (Fig. 3). Utilizing *jCE* (Prlić *et al.*, 2010), pairwise superposition of the structures of MHPCO (PDB entry 5hxi; Kobayashi *et al.*, 2017) and HbpA (PDB entry 4cy8; Jensen *et al.*, 2015) with PobA from *P. putida* showed that MHPCO and HbpA are structurally similar, with Z-scores of 7.0 and 6.4, respectively [Figs. 5(a) and 5(b)]. Although the active site and the FAD-binding site do not have conserved residues in these flavoprotein oxygenases, common secondary-structure elements that are essential for catalysis, such as the $\beta\alpha\beta$ fold, β -sheet wall and $\alpha 12$ helix, are present. These results suggest that as flavoproteins evolve for specific metabolic functions, core secondary structures involved in the binding and electron transfer of FAD are essential for the enzyme to retain its catalytic capability as a monooxygenase.

4. Conclusions

In this study, we report the first crystal structure of the pHBH enzyme PobA from *P. putida* in complex with FAD and show that it is very similar when compared with pHBH structures from other *Pseudomonas* species. Furthermore, the portions of the residues required for substrate and FAD binding are identical, including the $\beta\alpha\beta$ fold and β -sheet wall found in other flavoprotein oxygenases. Considering the strong structural similarities between PobA from *P. putida* and the PHBHs from more pathogenic *Pseudomonas* species (*P. fluorescens* and *P. aeruginosa*), PobA could possibly serve as a candidate for a drug target to combat tetracycline resistance. These similarities are likely to be owing to the ability for plasmid-based horizontal gene transfer among the three species (Yomoda *et al.*, 2003). Future work in combatting tetracycline resistance should attempt to develop drugs targeting PobA and to measure off-target effects in the gut microbiome.

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