Structural Characterization of *Pandoraea pnomenusa* B-356 Biphenyl Dioxygenase Reveals Features of Potent Polychlorinated Biphenyl-Degrading Enzymes

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Abstract

The oxidative degradation of biphenyl and polychlorinated biphenyls (PCBs) is initiated in *Pandoraea pnomenusa* B-356 by biphenyl dioxygenase (BPDO<sub>B356</sub>). BPDO<sub>B356</sub>, a heterohexameric (2β)₃ Rieske oxygenase (RO), catalyzes the insertion of dioxygen with stereo- and regioselectivity at the 2,3-carbons of biphenyl, and can transform a broad spectrum of PCB congeners. Here we present the X-ray crystal structures of BPDO<sub>B356</sub> with and without its substrate biphenyl 1,6-Å resolution for both structures. In both cases, the Fe(II) has five ligands in a square pyramidal configuration: H233 N₁, H239 Nₑ, D386 O₁ and O₂, and a single water molecule. Analysis of the active sites of BPDO<sub>B356</sub> and related ROs revealed structural features that likely contribute to the superior PCB-degrading ability of certain BPDOs. First, the active site cavity readily accommodates biphenyl with minimal conformational rearrangement. Second, M231 was predicted to sterically interfere with binding of some PCBs, and substitution of this residue yielded variants that transform 2,2'-dichlorobiphenyl more effectively. Third, in addition to the volume and shape of the active site, residues at the active site entrance also apparently influence substrate preference. Finally, comparison of the conformation of the active site entrance loop among ROs provides a basis for a structure-based classification consistent with a phylogeny derived from amino acid sequence alignments.

Introduction

Polychlorinated biphenyls (PCBs) are among the most pervasive and persistent chlorinated environmental pollutants despite long-term regulation of their manufacture and use [1]. The discovery that many bacterial strains are able to at least partially degrade PCBs has fueled research directed toward improving bioremediation strategies to clean-up contaminated sites. In aerobic bacteria, PCBs are degraded co-metabolically by enzymes of the biphenyl (Bph) pathway [2]. The first four Bph enzymes comprise a typical meta-cleavage pathway involving the initial generation and subsequent ring fission of a catecholic metabolite. Bacterial strains vary widely in their abilities to degrade PCBs. However, the most potent PCB-degrading organisms, exemplified by *Burkholderia xenovorans* LB400 [3], *Rhodococcus jostii* RHA1 [4] and *Pandoraea pnomenusa* B-356 (formerly *Comamonas testosterone* B-356 [5]), are able to transform congeners containing up to 7 chloro substituents.

Biphenyl dioxygenase is the first enzyme of the Bph pathway and the major determinant of PCB degradation [2]. Indeed, the reported PCB-degrading abilities of bacterial isolates largely reflect the PCB-transforming potency of their biphenyl dioxygenase. The catalytic component of this enzyme is a Rieske-type oxygenase (RO), which catalyzes the highly regio- and stereoselective insertion of dioxygen into an aromatic ring, activating the latter for subsequent catabolism. In addition to the oxygenase component (BphAE or BPDO), biphenyl dioxygenase incorporates an FAD-containing reductase (BphG) and a “Rieske-type” ferredoxin
Crystal Structure of a Potent PCB Degrading Enzyme

Results

Crystallization of BPDOB356
BPDOB356 crystals were grown in an anaerobic environment (≤2 ppm O2) to maintain the oxidation state of the iron centers. The characteristically reddish-brown colored crystals exhibited a rhombic morphology and belonged to the space group P43. The asymmetric unit contains one αβ protomer (V_m = 2.7 Da/Å^3) and the best crystals diffracted to 1.5 Å resolution.

Crystallographic refinement and final model
The final models include the complete β-subunit, but lack the 18 N-terminal residues of the α-subunit, which were never represented in the electron density and were assumed to be present and disordered. All structures were refined to between 1.5 and 1.6 Å resolution with final R_{free} and R_{free} values less than 20%. Additional data and statistics are presented in Table 1.

Quaternary Structure of BPDO and Phylogenetic Analysis
BPDOB356 is an (αβ)_3 heterohexamer, which is similar to previously reported structures of ROs, such as NDO9816-4 [6] (Figure 2). The heterohexameric ROs have very similar three-dimensional structures with rmsds between 0.7–2.4 Å for the Cz atoms of the α-subunits and between 0.7–1.3 Å for the β-subunits. The superposition of eight α-subunit crystal structures was used to generate a structure-based alignment profile used to guide the overall alignment of amino acid sequences for 25 homologous ROs. The phylogenetic tree generated using this alignment displays three distinct groups (Figure 3). The available functional data indicate that these groups reflect the substrate specificities of the ROs. For example, Group II contains ROs responsible for hydroxylating nitro-containing aromatics, such as NbzA [23], and Group III contains ROs responsible for hydroxylating phthalate, such as PhtA [24]. In Group I, the potent PCB degrading enzymes BPDOB356 and BPDOB1 hetero-octamers together with cumene dioxygenase of Pseudomonas putida (CumDO) and are distinct from the cluster of benzene dioxygenases that include BPDOB1 [8].

Our revised classification based on crystal structure-based sequence alignments and reliance on functional data, while similar to the scheme developed by Nam and coworkers, which was based only on sequences [24], adds a new group to their classification. Whereas our Group I corresponds quite well with their Group IV, our classification divides their Group III into two groups presented as Group II and Group III in Figure 3. More recently, Kweon et al. reported an inclusive classification of Rieske oxygenase systems driven by primary sequence data and encompassing all protein components involved in electron delivery and catalysis [25]. Although the present approach and that used by Kweon et al. are distinctly different, the molecular phylogenies conform: Group I in Figure 3 maps to Type IV of Kweon et al., Group II maps to Type III, and Group III maps to Type V.

Structure of the β-subunit
Despite the global similarity of the known β-subunit structures, as demonstrated by the overall low rmsds of 0.7–1.3 Å (Ca atoms), distinctive structural features divide the structures into two categories, similar to those found based on the phylogenetic analysis of the α-subunit: those that resemble NDO9816-4 (Group II) and those that resemble BPDOB356 (Group I). The fold and interactions of the N-terminal residues with a neighboring β-subunit constitute one of the differentiating features. In NDO, these residues form a two-turn α helix, which interacts with helix 2_b (α2_b) and α3_b of an adjacent β-subunit (Note: Elements of secondary structure are numbered sequentially with separate lists.
for helices and \( \beta \) strands in each subunit. Subscripts \( \alpha \) and \( \beta \) identify the subunit; \( \alpha_2 \) is the second helix along the chain of the \( \beta \)-subunit. Residues are identified by the one-letter amino acid code with the residue number appended; when necessary, the subunit is indicated by a subscripted \( \alpha \) or \( \beta \). In BPDOB356, the observed residues meander across the outer surface of the central sheet of the neighboring \( \beta \)-subunit, interacting with and covering residues that are solvent-exposed in NDO.

Other differentiating features occur in loops that interact with the \( \alpha \)-subunit. The loop connecting strands \( \beta_1 \) and \( \beta_2 \) packs more extensively against the Rieske domain in NDO\_9816-4 than in BPDOB356. There is also variation in the loop connecting \( \beta_5 \) and \( \beta_6 \), which bends towards \( \alpha_1 \) in BPDOB356 and away from it in NDO\_9816-4. The structures of the \( \beta \)-subunits of BPDO\_RHA1 and CumDO\_IP01 are more similar to BPDOB356, while that of nitrobenzene dioxygenase\_JS765 (NBDO\_JS765), polyaromatic hydrocarbon ring-hydroxylating dioxygenase\_CHY1 (RHDO\_CHY1), BPDOB1, and NDO\_12308 are more similar to NDO\_9816-4, which is consistent with sequence-based phylogeny based solely on the \( \alpha \)-subunit (Figure 3).

The BPDOB356 \( \beta \)-subunit \( \alpha_3 \)-\( \beta_3 \) loop participates in a web of hydrogen bonds with side chains from \( \alpha_1 \) including Q384 and D385, the residues immediately preceding the active site Fe ligand D386. These interfacial interactions may couple \( \alpha \) and \( \beta \) in a way that directly affects the ability of the active site to adjust to different substrates. Compared to BPDOB356, the corresponding loop in NDO\_9816-4 is approximately 3.0 Å further away from the \( \alpha \)-subunit, is not involved in a similarly extensive hydrogen-bonding network, and might not be expected to exert a similar influence on the adaptations of the active site during catalysis.

### Table 1. Refinement parameters and statistics.

| Model Content (non-hydrogen atoms) | BPDOB356 MES buffer | BPDOB356-biphenyl MES buffer |
|------------------------------------|----------------------|-----------------------------|
| Protein atoms                      | 5015                 | 5015                        |
| protein atoms in alternate conformations | 105                 | 80                          |
| Fe(III), Fe(II) & S\(^2\) atoms    | 5                    | 5                           |
| water oxygen atoms                 | 582                  | 640                         |
| Diffraction Data                   |                      |                             |
| resolution range (Å)               | 10.1.63              | 80.1.58                     |
| number of reflections              | 86168                | 91502                       |
| twin fraction (%)                  | 0.36                 | 0.02                        |
| \( R_{\text{refined}} \) (%)       | 11.7                 | 17.8                        |
| \( R_{\text{free}} \) (%)          | 16.6                 | 19.8                        |
| Average \( B \) values (Å\(^2\))   |                      |                             |
| protein atoms (main, side chain)   | 12.1, 15.6           | 22.4, 22.6                  |
| Fe(III), Fe(II) & S\(^2\) atoms   | 10.5, 17.8, 11.0     | 17.8, 26.8, 18.5            |
| water oxygen atoms                 | 32.5                 |                             |
| all atoms                          |                      |                             |
| Rms deviations from restraints     |                      |                             |
| bond lengths (Å)                   | 0.016                | 0.008                       |
| bond angles (°)                    | 1.72                 | 1.12                        |

Crystal Structure of a Potent PCB Degrading Enzyme

**Figure 2. Ribbon diagram showing the overall structure of BPDOB356.** Two orthogonal views showing three \( \alpha/\beta \) protomers arranged around the crystallographic three-fold axis to form the active hexamer. This arrangement allows the Rieske domain (green ribbons) from the tan \( \alpha \)-subunit to interact with the catalytic Fe\(^2+\) (rust sphere) in the adjacent subunit (purple ribbons). All structural graphics were created using Pymol (www.pymol.org).

doi:10.1371/journal.pone.0052550.g002
Crystal Structure of a Potent PCB Degrading Enzyme

Figure 3. Unrooted phylogenetic tree obtained from a crystal structure-based sequence alignment of 25 α-subunits of related Rieske Oxygenases. Bootstrap values out of 100 replicates are indicated. The proteins are abbreviated using the gene name and strain as follows: biphenyl dioxygenase from Pandoraea promenusa B-356 (BphA356, GenPeptID: AAC45256); Burkholderia xenovorans LB400 (BphA1-LB400, GenPeptID: YP_556400); Rhodococcus globulus P6 (BphA1-P6, GenPeptID: CAAS6346); Rhodococcus jostii RHA1 (BphA1-RHA1, GenPeptID: BAA06868), and Sphingobium yokohamae B1 (BphA1-B1, GenPeptID: ABM91740); benzene dioxygenase from Pseudomonas putida ML2 (BedC1-ML2, GenPeptID: Q07944); benzoate dioxygenase from P. putida CAG17576) (EbdA1RHA1, GenPeptID: ABG99212); 3-phenylpropionate dioxygenase from Sphingobium yanoikuyae Pseudomonas abietaniphila Rhodococcus globerulusYP_556409), P. putida CAG17576; dibenzofuran dioxygenase from Terrabacter AAL50021); dibenzo-p-dioxin dioxygenase from Comomonas benzoate dioxygenase from P. putida CAG17576); and tetraline dioxygenase from Sphingomonas macrogoltabidus (EbdA1RHA1, GenPeptID: BAC92718); cumene dioxygenase from Pseudomonas obietaniphila BKME (DitA9BKME, GenPeptID: AAD21063); dinitrotoluene dioxygenase from Burkholderia cepacia R34 (DntAC34, GenPeptID: AAL50021); dibenz-p-dioxin dioxygenase from Sphingomonas (DnxA1-ML2, GenPeptID: CAAS6346), ethylbenzene dioxygenase from R. jostii RHA1 (EbdA1RAHA1, GenPeptID: BAC92718); 3-phenylpropionate dioxygenase from E. coli K-12 (HcaE12, GenPeptID: ACB03690); naphthalene dioxygenase from Rhodococcus sp. NCIMB 12038 (NarA12038, GenPeptID: AAD28100) and Pseudomonas putida 9816-4 (NdoB9816, GenPeptID: P0A110); nitrobenzene dioxygenase from Comamonas sp. J5765 (NdoB1-J5765, GenPeptID: AAL76202); polyaromatic hydrocarbon dioxygenase from Mycobacterium vanbaalenii PYR-1(NidA1PYR1, GenPeptID: AAF75991, NidA3PYR1, GenPeptID: AAY85176) and Burkholderia sp. RP007 (PhnA1RP07, GenPeptID: AAD09872); phthalate dioxygenase from R. jostii RHA1 (PadA1RHA1, GenPeptID: ABG99212) and Burkholderia sp. D8F63 (PhnA1DF83, GenPeptID: BAC534156); phenanthrene dioxygenase from Nocardioaves sp. K7 (PhnA1K7, GenPeptID: AAD09872); and tetraline dioxygenase from Sphingomonas macrogoltabidus TFA (ThnA1TFA, GenPeptID: AAN26443). Proteins for which the crystal structure was used for alignment are indicated in bold text in the figure. doi:10.1371/journal.pone.0052550.g003

Thus, variations in interactions at this interface could explain previously reported inconsistencies in substrate-profiling experiments based on limited mutagenesis or subunit exchange to probe the role of the β-subunit.

Several studies of α₁β₁ chimeric enzymes have established that the β-subunit plays a role in determining substrate specificity in ROs [26–28]. In studies of the BPDO B356, the Fe-S cluster is linked to the mononuclear Fe(II) by a hydrogen bonding network comprised of cluster ligand H123 of one α-subunit with the Rieske Fe 2S2 cluster serving as an electron donor during the catalytic cycle (Figure 2).

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Crystal Structure of a Potent PCB Degrading Enzyme

are quite similar with catalytically important residues conserved in structurally equivalent positions.

The most dramatic of the structural variations involves residues of low sequence identity (Figure S1) corresponding to 249–262 in BPDOB356, which form the entrance to the active site. Other differences are found in the extended helix (α13α) and the C-terminal region, where NDO9816-4, NBDOB356, RHDOCHY-1, and BPDOB1 have an extended helical tail.

Coordination of the Mononuclear Fe

BPDOB356 was purified anaerobically with Fe(II) in the mononuclear Fe site from the addition of (NH4)2Fe(SO4)2·6H2O during purification and crystallization with the crystals being flash-frozen while maintaining anaerobic conditions [17]. Previously, this as isolated BPDOB356 was determined to have an oxidized Rieske cluster prior to data collection, and qualitatively demonstrated X-ray-induced reduction after data collection (data not shown). The as isolated mononuclear Fe site in BPDOB356 exhibits square pyramidal coordination by two histidines, a bidentate aspartate, and a water molecule. The BPDOB356 structures demonstrate 2.2 Å bonds to the Nε2 of both H233 and H239, as well as bidentate binding to O61 (2.2 Å) and O82 (2.4 Å) of D386 (Figure 4). A single water ligand (W1) at a distance of 2.0 Å is observed (Figure 4a).

The coordination sphere of the mononuclear iron is remarkably similar in the BPDOB356:biphenyl complex. As in the substrate-free enzyme, the iron is pentacoordinate and of square pyramidal geometry (Figure 4b). H239 is the axial ligand, and the Fe is displaced toward it out of the basal plane by 0.5 Å. The Fe(II) coordination and geometry is thus similar to a variety of non-RO enzymes that coordinate Fe with histidines and carboxylic acids [31–34].

Structural Influences on Substrate Preference

The structural analyses suggest that differences both at the entrance to and within the active site cavity of the α-subunit likely contribute to differences in substrate preferences among ROs. In BPDOB356, access to the mononuclear Fe is via a 20 Å L-shaped tunnel (Figure 5a,c). This entrance is formed by residues 235–237, α7γ residue 240, α7γβ17γ loop residues 253–259γ, and β24γα313α loop residue 431γ.

Residues analogous to the BPDOB356 loop residues 253–259γ are key components of the active site entrance in all ROs. The location and form of key entrance loop residues are similar for BPDOB356 and CumDOIP01, and both correspond to the “loop 1” conformation defined for CumDOIP01 [35]. In contrast, for the ROs clustered with NDO9816-4 [6], the equivalent loop residues have very different conformation, called “loop 2” by Dong and co-workers [35], where the loop is located at the opposite side of the active site entrance. Thus, different phylogenetic clusters of ROs may use corresponding loop residues differently to regulate access to the active site. Although the entrance residues are disordered in BPDOB1HA1, the phylogenetic analysis predicts its association with the “loop 1” cluster, and a surface rendering of BPDOB1HA1 (Figure 5c) confirms that its entrance and cavity are more similar to BPDOB356 (Figure 5c) than NDO9816-4 (Figure 5d).

In summary, we predict that the entrance loop favors the “loop 1” conformation of BPDOB1HA1 and in all enzymes within the phylogenetic cluster that includes BPDOB356 and CumDOIP01.

Comparison of the active site cavities of BPDOB356-type (Group I) and NDO9816-4-type (Group II) ROs reveals further differences. For both types, the cavity can be divided into two subsites: a proximal (P) subsite, which binds the ring that is subject to hydroxylation, and the distal (D) subsite, which binds the second ring of biphenyl in the case of BPDO. For BPDOB356, the P subsite is lined by side chains of Q226, F227, H233, H321, L331, and the carbonyl of D230; whereas the distal pocket is lined by residues M231, A234, H239, F277, L284, G319, I334, F376, and F382. Amongst the BPDOB356 cluster of ROs, residues lining the P subsite are invariant, while there are only conservative substitutions among the residues lining the D subsite. F277, I283, V287, G319, and F382 in BPDOB356 correspond to F278, L284, I288, A321, and Y384 in CumDOIP01 and to Y268, L274, I278, A311, and F374 in BPDOB1HA1.

With respect to three-dimensional structure, the active site cavities of the various ROs might be compared by semi-quantitative measurements of volume or by assessment of shapes and surface features; the latter appearing seems to be the most revealing approach. For example, Figure 5 compares surface and volume renderings of the active site cavities of BPDOB356 (Figure 5c) and NDO9816-4 (Figure 5d) and reveals that the cavity of BPDOB356 is distinctly bicornuate, whereas that of NDO9816-4 appears unicornuate and lacks free space distal to the Fe; thus the

Figure 4. 2Fo-Fc electron density maps (contoured at 1σ above the mean) showing the coordination geometry of the mononuclear Fe2+. a) The mononuclear Fe2+ geometry found in the crystal structure determined in the presence of MES buffer, and b) in the presence of MES buffer and biphenyl. The Fe atom is coordinated by two histidines, a bidentate aspartate, and one water molecule (W1). doi:10.1371/journal.pone.0052550.g004
NDO9816-4 cavity appears relatively flat, consistent with the shape of naphthalene. For other ROs, the planarity or non-planarity of the substrates is consistent with the architecture of their active sites. The active site of NBDO9816-4 accommodates a planar substrate. On the other hand, the active site architecture of CumDOIP01 (Figure 5f), which presumably catalyzes the oxidation of a planar substrate, is less pronounced than that of BPDOB356. The view of the active site cavity of d) NDO9816-4 and g) NDO12038 has been rotated slightly relative to that of BPDOB356 in order to provide an unobstructed view of the entrance passageway.

doi:10.1371/journal.pone.0052550.g005

Figure 5. The positions and surface representations of the active site invaginations of BPDOB356, NDO9816-4, BPDOB916A1, CumDOIP01, and NDO12038. 

a) Shows the overall active site cavity of BPDOB356 as determined by the program VOIDOO. b) The overall active site cavity of NDO9816-4 determined similar to a). The solvent accessible surface representations calculated by the program Pymol for c) BPDOB356, d) NDO9816-4, e) BPDOB916A1, f) CumDOIP01, and g) NDO12038. BPDOB356 has a much larger active site cavity relative to BPDOB916A1. The distal pocket of CumDOIP01 is less pronounced than that of BPDOB356. The view of the active site cavity of d) NDO9816-4 and g) NDO12038 has been rotated slightly relative to that of BPDOB356 in order to provide an unobstructed view of the entrance passageway.

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of cumene, a molecule distinctly nonplanar although similar in size to nitrobenzene, is similar to that of BPDOB356.

A further comparison of the active sites of BPDOB356 and CumDOIP01 is also of interest. The source bacterial strain for CumDOIP01 can co-metabolize, but not grow on, biphenyl [36]. This preference for cumene versus biphenyl may be explained by an obstruction of the D subsite by residue A321 of CumDOIP01, thereby creating a smaller cavity (Figure 5f) than in BPDOB356 (Figure 5c) with its structurally analogous G319.

Comparison of the active sites of BPDOB356 and BPDOB101 in substrate free and biphenyl bound states reveals a difference that is likely to be important in the context of ability to process larger substrates and a wide range of PCBs. In the BPDOB356-biphenyl complex, the 2,3-carbons are 4.3 Å from the Fe(II) and the dihedral angle between the two aromatic rings is 112° (Figure 4b). In the BPDOB101-biphenyl complex the 2,3-carbons are 4.5 Å from the Fe(II) and the dihedral angle is 124° [7]. Thus, within experimental error, the position and conformation of the substrate are not distinguishable and likely represent a productive binding mode. However, the adjustments in protein conformation that accompany binding of biphenyl are much less dramatic in BPDOB356 than in BPDOB101. In particular, upon binding of biphenyl to BPDOB356, the side chain torsion angles of I283 in the D subsite adjust slightly to move the C81 atom away from biphenyl. In contrast, upon biphenyl binding in BPDOB101, the C8 of the analogous residue, L274, shifts about 2 Å to withdraw the side chain from the D subsite. This movement is part of an overall shift of ~6 Å to accommodate biphenyl [7]. A requirement for large conformational changes to bind biphenyl may translate to lower reactivity of BPDOB101 towards substrates larger than biphenyl. This hypothesis is consistent with the placement of BPDOB101 in a phylogenetic cluster occupied by ROs characterized as benzene transforming enzymes.

As a corollary, the less–restricted active site cavity of BPDOB356 may explain its greater reactivity to a broad spectrum of substituted biphenyls, such as PCB congeners [17,18]. Moreover, the arrangement of residues and overall dimensions of the BPDOB356 active site cavity may provide a structural explanation of why ROs that clustered with BPDOB356 include the well-characterized potent PCB degrading enzymes.

Mutagenesis and Steady-state Kinetics

The role of the conserved active site residue M231 on the selectivity of BPDOB356 was probed by site directed mutagenesis. M231A was chosen because of its unique location at the junction of the P and D subsites and the placement of the M231 S6 atom in the plane of the proximal ring of biphenyl and near an ortho carbon atom on that ring. Substitution of a chlorine at this ortho carbon position would result in a steric clash with the M231 S6 atom. Therefore, mutations were chosen to alter the steric limitations of the active site (M231A) as well as the polar influences of residues in the active site (M231T). Steady-state kinetic characterization of these two variants, M231A and M231T, were performed with biphenyl and 2,2'-dichlorobiphenyl substrates (Figure 6). M231A and M231T each showed Michaelis-Menten kinetics for the dependence of the initial rate of oxygen consumption on the concentration of biphenyl. Substituting M231A with either smaller side chain lowered the apparent specificity of the enzyme for both biphenyl (4- to 6-fold) and 2,2'-dichlorobiphenyl (~3-fold; Table 2). In the event of unproductive catalytic turnover or uncoupling H2O2 would be generated during the assay and the addition of catalase was used to determine the amount generated. Hydroxylation of biphenyl was well-coupled to O2 consumption in the variants. Interestingly, the transformation of the dichlorobi-phenyl was better coupled to O2 consumption in the variants as compared to wild-type enzyme. In previous studies uncoupling was not detected for wild-type enzyme with biphenyl as substrate [17].

Discussion

Here we present the crystal structures of BPDOB356 at 1.6 Å resolution, and BPDOB356 in complex with its substrate biphenyl at 1.6 Å resolution. BPDOB356 is a typical heterohexameric RO with 2β protomers arranged about a three-fold symmetry axis to place the Rieske Fe2S2 cluster of each 2-subunit within ~12 Å of the mononuclear Fe(II) of an adjacent 3-subunit.

The coordination state of the active site Fe has been a significant focus of research on ROs. Prior studies indicate that the redox state of the Rieske center plays an important role in modulating the coordination environment of the mononuclear Fe site. For example, the crystal structure of OxoDO showed that the Fe(II) center changed from a pentacoordinate state with a single water ligand to a hexacoordinate state with two water ligands when the crystals were grown in the presence of dithionite [37]. Similarly, side-on binding of O2 established a hexacoordinate Fe(II) in NDO8164 after dithionite reduction of the protein [38]. In contrast, BPDOB356 used in the presented study was purified under anaerobic conditions [17], and crystals were subsequently grown and frozen in an anaerobic environment, avoiding the use of strong reducing reagents. Therefore, the observed pentacoordinate state might represent a potential resting state of BPDOB356 with Fe(II) in the active site and an oxidized Fe2S2 cluster. Given the variability of sample treatment and observed coordination states, it remains unclear whether this state is exclusive to BPDOB356 or is a common state for all ROs.

Comparison of the active site cavities of BPDOB356, CumDOIP01, and BPDOB101 (Group I) to those of BPDOB11, NDO8164-RHDO and NDOB3765 (Group II) showed a fundamental distinction between these two groups. Within Group I the active site cavities of BPDOB356, CumDOIP01, and BPDOB101 can clearly be subdivided into two distinct subsites based on the invaginations of the active site, the P subsite where ring hydroxylation occurs, and the D subsite that accommodates the rest of the substrate. In contrast, the active site cavities of...
**Table 2.** Apparent steady-state kinetic parameters of BPDO<sub>B356</sub> wild-type (wt) and variants (M231A and M231T) for biphenyl and 2,2'-dichlorobiphenyl.

|            | Biphenyl         | 2,2'-Dichlorobiphenyl |
|------------|-----------------|-----------------------|
|            | \( k_m \) (\( \mu \text{M} \)) | \( k_{cat} \) (s<sup>-1</sup>) | \( k_{cat}/k_m \) (\( \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \)) | \( k_m \) (\( \mu \text{M} \)) | \( k_{cat} \) (s<sup>-1</sup>) | \( k_{cat}/k_m \) (\( \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \)) | \( \text{H}_2\text{O}_2/\text{O}_2 \) |
| WT         | 6.2 (0.5)       | 7.3 (0.2)             | 1.2 (0.1)                      | 1.1 (0.2) | 1.8 (0.1) | 1.7 (0.3) | 0.61 (0.03) |
| M231A       | 9.4 (1.9)       | 2.1 (0.1)             | 0.2 (0.05)                     | 5.7 (0.9) | 4.2 (0.2) | 0.7 (0.1) | 0.42 (0.06) |
| M231T       | 11.1 (3.1)      | 2.9 (0.3)             | 0.3 (0.1)                      | 6.4 (1.1) | 3.4 (0.2) | 0.5 (0.1) | 0.36 (0.03) |

Coupling parameters are given for 2,2'-dichlorobiphenyl only. Standard deviations are given in parenthesis.

*These values were reported in [17].

doi:10.1371/journal.pone.0052550.t002

NDO<sub>9816-4</sub>, NBDO<sub>9765</sub>, RHDO<sub>CHY-1</sub>, and BPDO<sub>B1</sub> from Group II contain no such clear divisions.

The structure of BPDO<sub>B356</sub> may illuminate active site structural factors required for potent PCB-degrading ROs in Group I. Although binding of biphenyl to BPDO<sub>B356</sub> required only minor adjustments by the protein, biphenyl binding to BPDO<sub>SHA1</sub> required extensive conformational changes that expand the active site. For CumDO<sub>HO1</sub>, a constriction of the active site cavity due to sequence variation may dictate a preference for cumene over larger potential substrates, such as biphenyl and PCBs. These observations may explain the reactivity of BPDO<sub>B356</sub> with a broad range of recalcitrant PCB congeners on the basis of facility of aromatic substrate binding alone. By extension, our comparative analysis of these active sites provides a structure-based explanation for the reactivity of related potent PCB degrading ROs such as BPDO<sub>B356</sub> and BPDO<sub>SHA1</sub>.

For dioxygenases, unhindered binding of the aromatic substrate has determining significance to the binding and activation of the dioxygen substrate. If binding of a particular aromatic substrate challenges the productive binding of dioxygen, the consequence could be increased uncoupling of electron consumption and oxygen activation from the desired reaction. The consequences of a highly uncoupled reaction are highly detrimental and include loss of reducing equivalents with release of reactive oxygen species, inhibition, and suicide inactivation [39].

Finally, as demonstrated by the effect of mutations at M231 on steady state kinetic parameters for the reaction with a representative ortho-chlorinated PCB, 2,2'-dichlorobiphenyl, we also showed that strategic alterations of the active site cavity based on the crystal structure can improve the processing of specific PCB congeners. The effects of the M231A and M231T mutations resulted in improvements in turnover number and coupling with the dichloro-substituted substrate, and are consistent with a more accommodating active site. This is further supported by a previous study of BPDO<sub>B1</sub>, where the corresponding Met to Ala conversion resulted in a variant with significantly altered regioselectivity with two substrates, 2,3'-dichlorobiphenyl and 3,3'-dichlorobiphenyl, but the effects on the kinetic parameters and coupling were not reported [40]. Thus, this structural information may contribute to strategies for the engineering of improved bioremediation pathways.

**Materials and Methods**

**Phylogenetic Analysis of Rieske Oxygenase Sequences**

Sequences used for the phylogenetic analysis were selected from ROs whose X-ray crystal structures have been determined with additional sequences selected from a subset of related sequences. A structure-based sequence alignment was first accomplished by pair-wise superpositions of proteins of known structure. Additional sequences were added and aligned using CLUSTALW [41].

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**Protein Purification and Directed Mutagenesis**

BPDO<sub>B356</sub> and its variants were heterologously produced and purified anaerobically as described previously for the wild-type RO [17]. Directed mutagenesis was performed using the QuickChange protocol (Stratagene) and the following oligonucleotides: 5'-GCAGTTCTCGAGCCGA CGGGTACCGCGGCGG-3' (M231A mutation) and 5'-GCAGTGTCGAGCCGACAGT ACCACGCGGCGG-3' (M231T mutation) combined with their reverse complements. PfuI DNA polymerase was used amplify the plasmids following annealing of the primers at 52°C.

**Crystallization**

Crystals were grown by sitting drop vapor diffusion under anaerobic conditions within a N<sub>2</sub> atmosphere glove box (Innovative Technologies, Newburyport, MA). Two protocols were used. Crystallization from a solution containing 100 mM sodium citrate, pH 5.8; 10% v/v 2-propanol; and 24% w/v PEG4000 at 20°C was described previously [17]. In the second protocol, protein (36 mg/ml) in 25 mM HEPES, pH 7.3; 2 mM DTT; 10% v/v glycerol; and 0.25 mM ferrous ammonium sulfate was diluted to 7 mg/ml by addition of a solution containing: 25 mM HEPES, pH 7.3; 10% v/v glycerol; 50 mM NaCl; and 0.25 mM ferrous ammonium sulfate. Crystals were obtained via sitting drop vapor diffusion methods by mixing 4 μl of protein solution with 4 μl of a reservoir solution containing: 100 mM MES, pH 6.0; PEG 4000 (18–28% w/v); 3.5 mM ferrous ammonium sulfate; and 16% v/v 2-propanol. In both cases, the best diffracting crystals grew in one to two weeks. In the first protocol, the citrate-buffered crystals were typically 0.3 mm ± 0.1 mm × 0.1 mm and belonged to the space group type P<sub>3</sub> with cell dimensions \( a = 36.5 \text{ Å} \), \( c = 107.0 \text{ Å} \) for the triply primitive hexagonal cell. Typical MES-buffered crystals were 0.3 mm ± 0.2 mm × 0.2 mm, and belong to the same space group with similar cell dimensions, \( a = 134.6 \text{ Å} \), \( c = 104.6 \text{ Å} \). The structure of the BPDO<sub>B356</sub>: biphenyl complex was obtained by adding a small amount of biphenyl powder to crystals and incubating for a period of 24 hours before harvesting.
Diffraction Experiments

Diffraction data were collected at cryogenic conditions (~100K) from crystals frozen in liquid nitrogen after brief incubation in a solution similar to the reservoir solution, but with the 2-propanol replaced by 20% v/v glycerol [17]. The diffraction data were indexed and reduced to averaged intensities using the HKL program suite [44]. Intensities were converted to structure factor amplitudes using programs from the CCP4 package [45]. Prior to diffraction experiments using synchrotron radiation, crystals were typically screened for quality of diffraction and the presence of twinning using Cu-Kα radiation from a Rigaku rotating anode X-ray generator equipped with mirror optics and an R-AXIS image plate area detector (Rigaku/MSD). High-resolution diffraction data used for refinement were collected at the Advanced Photon Source synchrotron (APS) using beamlines BM-14-C and SBC-19ID and are summarized in Table 3.

Detection and Analysis of Twinning

Twinning was detected by analysis of plots of the cumulative intensity distribution, N(z), [46,47], as produced by the program TRUNCATE [48]. The comparison of observed N(z) to the expected distribution coupled with the observation of a single lattice in the diffraction pattern indicated merohedral twinning. The twin fraction was assessed by analysis of the cumulative distribution of \( I_{\text{obs}}(h_1) - I_{\text{calc}}(h_2) / I_{\text{calc}}(h_1) + I_{\text{obs}}(h_2) \), where \( h_1 \) and \( h_2 \) are Miller indices related by the twinning operation [49], and varied from 0–50% for the crystals used in this study as reported for each crystal in Table 3.

Molecular Replacement and Model Building

The structure of BPDO_{B356} was determined by molecular replacement using naphthalene dioxygenase (PDB ID: 1NDO) as a search model. AMORE [50] was used to calculate the cross-rotation and translation functions. A dominant solution was obtained and used for rigid body fitting within AMORE, CNS [51] was used for further rigid body refinement and to calculate an initial map. The initial map was readily interpreted such that 534 residues (83%) of the BPDO_{B356} sequence were rapidly modeled using the program O [52].

Refinement of crystallographic models

Initial models for the structure of the citrate-buffered crystals were refined using the program CNS with automated parameter adjustment and electron density map calculations. Final models of BPDO_{B356} and the BPDO_{B356}-biphenyl complex for the structure of the MES-buffered crystals were refined using SHELX-97 [53] and REFMAC5 [54], respectively. O [52] was used for model building, electron density evaluation and superposition of models. Anomalous difference electron density maps were used to verify the presence of iron at the active site and to assess its occupancy by comparison to the density observed for iron in the Rieske cluster. Atomic models and structure factors have been deposited in the Protein Data Bank under the PDB IDs 3GZY (BPDO_{B356}) and 3GZX (BPDO_{B356}-biphenyl complex).

Steady-state Kinetic and Coupling Measurements

Enzyme activity was assayed by following O_2 consumption using a computer-interfaced Clark-type polarographic oxygen electrode essentially as described previously [17]. The standard reaction mixture contained 70 mM Fe(2SO_4)_2(NH_4)_2, 280 μM biphenyl, 125 μM NADH, 1.2 mM BphGB356, 2.8 mM BphFLB400 and 0.26 μM BPDOB356 in air-saturated 50 mM MES buffer, pH 6.0. Initial velocity measurements were taken using concentrations ranging from 0.9–176 μM 2,2′-dichlorobiphenyl (Note: 2,2′-dichlorobiphenyl is a suspected cancer hazard and as described in the MSDS appropriate personal protection equipment and handling measures were followed). Coupling between O_2 consumption and biphenyl turnover was estimated by adding catalase to the assay 90 s after initiating the reaction. The amount of O_2 released was taken to reflect 50% of the hydrogen peroxide produced.

Supporting Information

Figure S1 Sequence alignment showing low sequence identity in the region that defines the active site entrance to BPDO_{B356}.

Acknowledgments

We would like to thank the staff of BioCARS and SBC for their assistance with the X-ray diffraction experiments.
Author Contributions
Conceived and designed the experiments: CC NA PK JP LE JB. Performed the experiments: CC NA PK MC SCS. Analyzed the data: CC

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