Title: The structural basis of the enhanced pollutant-degrading capabilities of an engineered biphenyl dioxygenase

Running Title: Structural Studies of BphAE variant II9

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Abbreviations used: DDT- dichlorodiphenyltrichloroethane; BPDO- biphenyl dioxygenase; PCB- polychlorinated biphenyl, bph- biphenyl
Abstract

Biphenyl dioxygenase, the first enzyme of the biphenyl catabolic pathway, is a major determinant of which PCB congeners are metabolized by a given bacterial strain. Ongoing efforts aim to engineer BphAE, the oxygenase component of the enzyme, to efficiently transform a wider range of congeners. BphAE-II9, a variant of BphAE-LB400 in which a seven-residue segment, TFNNIRI, has been replaced by the corresponding segment of BphAE-B356, GINTIRT, transforms a broader range of PCB congeners than either BphAE-LB400 or BphAE-B356, including 2,6-dichlorobiphenyl, 3,3'-dichlorobiphenyl, 4,4'-dichlorobiphenyl, and 2,3,4'-trichlorobiphenyl. To understand the structural basis of the enhanced activity of BphAE-II9, we have determined the three-dimensional structure of this variant in substrate-free and biphenyl-bound forms. Structural comparison with BphAE-LB400 reveals a flexible active site mouth and a relaxed substrate binding pocket in BphAE-II9 that allow it to bind different congeners, and which could be responsible for the enzyme’s altered specificity. Biochemical experiments revealed that BphAE-II9 transformed 2,3,4'-trichlorobiphenyl and 2,2',5,5'-tetrachlorobiphenyl more efficiently than BphAE-LB400 and BphAE-B356 did. BphAE-II9 also transformed the insecticide DDT more efficiently than either parental enzyme (apparent \( k_{\text{cat}}/K_m = 2.2 \pm 0.5 \) vs. 0.9 ± 0.5 mM\(^{-1}\)s\(^{-1}\) for BphAE-B356). Docking studies of the enzymes with these three substrates provide insight into the structural basis of the different substrate selectivity and regiospecificity of the enzymes.

Importance

Biphenyl dioxygenase is the first enzyme of the biphenyl degradation pathway that is involved in degradation of polychlorinated-biphenyls. Attempts have been made to identify the residues that influence the enzyme activity for the range of substrates among various species. In this study we have done the structural study of one variant of this enzyme that was produced by family shuffling of genes from two different species. Comparing the structure of this variant with the parent enzymes has
provided an important insight towards the molecular basis for the broader substrate preference of this enzyme. The structural and functional details gained in this study can be utilized to further engineer desired enzymatic activity, producing more potent enzymes.

Introduction

The microbial degradation of environmental pollutants has been extensively studied over the past few decades (1-3). Some pollutants, including polychlorinated biphenyls (PCBs), pose a threat to human health and to the biosphere due to their toxicity and persistence. Apprehension about PCBs led to prohibition of their production and use as well as regulation of their disposal and/or remediation (3-7). Bacterial degradation plays a pivotal role in bioremediation of PCB-contaminated soil and water (8-10). Aerobic degradation involves the biphenyl (Bph) catabolic pathway, which includes four enzyme-catalyzed reactions. Bacteria can utilize the Bph pathway to co-metabolize a variety of PCB congeners with differential effectiveness and different PCB congeners are transformed in a strain-dependent fashion (11). Till now, structural studies have been done for all four enzymes to understand the detailed mechanism that underlies this pathway (12-17).

Biphenyl dioxygenase (BPDO), the first enzyme of the Bph pathway, is a three-component ring-hydroxylation Rieske-type oxygenase (RO) that utilizes NADH and O₂ to transform biphenyl to cis-(2R,3S)-dihydro-dihydroxybiphenyl, Fig 1(A). The three components are a two-subunit oxygenase (BphAE), a ferredoxin (BphF), and a ferredoxin reductase (BphG) (18, 19). BphF and BphG deliver electrons from NADH to the oxygenase. As is typical of ring-hydroxylation ROs, the oxygenase is a three-fold symmetric heterohexamer assembled from three larger BphA (α) subunits and three smaller BphE (β) subunits such that the overall shape resembles that of a mushroom with the three α subunits forming the cap and the three β subunits forming the stem. Each α-subunit includes a [2Fe-2S] Rieske-
type cluster (His2Cys2 ligation) involved in electron transfer from external reductants to an active site containing a mononuclear Fe^{2+} ion coordinated by two His residues, an Asp residue (His233, His239, and Asp388 in BphAE_{LB400} (12)), and a conserved water molecule. The 3-fold symmetric arrangement of αβ dimers brings the [2Fe-2S] cluster of one dimer close to the active site of another, such that the Rieske center of one α-subunit is 12 Å from the Fe^{2+} atom of the neighboring subunit. It is generally accepted that electron transfer from the Rieske cluster to the Fe^{2+} occurs through a network of hydrogen bonds and Asp230 plays a major role in connecting the sites (20, 21).

The enzymatic capabilities of BPDO have been studied extensively because its substrate preference and the nature of the reaction products are major determinants of the transformations of different PCBs by the Bph pathway. For the purpose of bioremediation, it is desirable to expand the range of PCB congeners transformed by the Bph pathway. Accordingly, an extensive effort in enzyme engineering has been directed toward broadening the substrate range of BPDO. For example, relatively unbiased directed evolution has been used to alter the substrate profiles of variants derived from the closely related BPDOs of *Burkholderia xenovorans* LB400 and *Pseudomonas pseudoalcaligenes* KF707, two bacterial strains that degrade PCBs relatively well (22, 23). An alternative, more targeted strategy took into account comparisons of amino acid sequences and functional properties by Mondello *et al*., which identified four regions (I-IV) in the C-terminal domain of BphA that modulate the regioselectivity and regiospecificity of the enzyme (24). By shuffling the segment of *bphA* encoding the C-terminal portion of the *B. xenovorans* LB400 and *Pandoraea pnomenusa* B356 enzymes, Barriault *et al*.

Several of the most potent PCB-transforming BphAE variants have been derived from BphAE_{LB400} (26-28). BphAE_{B356} is a variant of BphAE_{LB400} in which the seven residues of Region III, 335TFNNIRI341, are replaced by the corresponding residues of BphAE_{B356}, 333GINTIRT339. Among
other characteristics, BphAE_{II9} is able to transform 2,3,4′-trichlorobiphenyl more efficiently than either parental enzyme (29). BphAE_{II10} adds a single residue substitution to the BphAE_{II9} background: Ala267 is replaced by Ser as occurs in BphAE_{B356}. Interestingly, the substrate range of BphAE_{II10} is significantly narrower than that of BphAE_{II9}. Finally, BphAE_{P4} was created by substituting two residues in Region III of BphAE_{LB400}: T335A and F336M (26). In liquid culture depletion assays, this variant eliminates several congeners better than BphAE_{LB400} does, including the very persistent 2,6-dichlorobiphenyl (26). Structural analysis revealed that the altered substrate specificity of BphAE_{P4} is associated both with changes in direct side chain-substrate interactions and with changes in residue-residue interactions near the active site; the latter appear to relieve constraints on the induced fit between the enzyme and substrates (12).

In the current study, we analyzed the crystal structure of BphAE_{II9} in the absence and presence of biphenyl to elucidate the molecular basis for the broader substrate preference of the enzyme. We focused on Region III residues to better understand how their interactions with other residues modulate substrate competence and reaction regiospecificity. Further, we report the results of biochemical assays and docking experiments with selected chlorinated biphenyl analogs and the insecticide 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (also known as dichlorodiphenyltrichloroethane, DDT) directed toward identification of the structural features in BphAE_{II9}, BphAE_{LB400}, and BphAE_{B356} responsible for the differential ability of the enzymes to transform these compounds.

**Experimental Procedures**

**Manipulation of DNA and preparation of BphAE_{II9} protein**

The DNA for BphAE_{II9}, a variant of BphAE_{LB400}, was obtained in a previous study by shuffling targeted regions of the \( bphA_{LB400} \) and \( bphA_{B356} \) genes (25). Gene \( bphAE_{II9} \) DNA was cloned in pET14b.
and transformed into *Escherichia coli* C41(DE3). BphAE$_{II9}$ was produced in that strain as a His-tagged recombinant protein and purified by affinity chromatography according to protocols published for BphAE$_{LB400}$ (27).

**Crystallographic Procedures**

Crystallization experiments were performed at 21°C in a glove box (Innovative Technologies, Newburyport, MA) with a circulating N$_2$ atmosphere (<5 ppm oxygen). Good quality, diffracting crystals were obtained from BphAE$_{II9}$ by sitting drop vapor diffusion using a well solution of 20 – 25% PEG 8000, 6% glycerol, 50 mM NaCl, and 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0. Crystals of the biphenyl-bound form of BphAE$_{II9}$ were obtained by adding a small amount of powdered biphenyl (purchased from Sigma-Aldrich [http://www.sigmaaldrich.com](http://www.sigmaaldrich.com)) directly to crystallization drops containing BphAE$_{II9}$ crystals and incubating for 30 minutes before harvesting the crystals. Diffraction data from crystals flash frozen by immersion in liquid N$_2$ were collected at the BioCARS 14BM-C beamline at the Advanced Photon Source (Argonne National Laboratories). Data were acquired for both substrate-free and biphenyl-bound crystals. The diffraction data were indexed, integrated, and scaled using the HKL2000 program suite. Initial phases were obtained by molecular replacement using MOLREP from the CCP4 v.6.3.0 software suite (30, 31). The crystal structure of a single αβ heterodimer of BphAE$_{LB400}$ (PDB ID: 2XRX) was used as a search model. REFMAC 5.2 (32) was used for rigid body refinement of the molecular replacement model and for subsequent rounds of restrained atomic parameter refinement. The program COOT was used for analysis of electron density maps and model building (33). Solvent molecules, Fe ions (for the Rieske clusters and mononuclear Fe$^{2+}$), and biphenyl molecules were added where the $F_o - F_c$ map had features of appropriate volume above 3σ and the $2F_o - 2F_c$ map showed density at 1σ. The
stereochemical properties of the refined models were evaluated using the program MOLPROBITY (34). The data collection, refinement statistics, and model quality for the two structures are summarized in Table 1. All molecular figures were prepared using the program PyMOL (35).

Assays and kinetic studies of BPDO from wild type and variant

DDT (99% pure) was obtained from Sigma-Aldrich, 2,3,4′-trichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl were obtained from Ultra Scientific. The ability of BphAE_{LB400}, BphAE_{B356}, and BphAE_{B9} to metabolize 2,3,4′-trichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl and DDT was assessed using BPDO systems reconstituted from His-tagged components produced and purified as described previously (12). The enzyme assays were performed in 200 μl of solution containing 100 to 800 nmol substrate and buffered with 50 mM MES, pH 6.0 at 37°C as described previously (36). Metabolites were identified by GC-MS analysis after the reaction medium was incubated for 15 min. The metabolites were extracted at pH 6.0 with ethyl acetate, then treated with n-butylboronate (nBuB) prior to GC-MS analysis, as described previously (37) using a Hewlett Packard HP6980 series gas chromatograph interfaced with an HP5973 mass selective detector (Agilent Technologies). To obtain kinetic parameters with DDT as substrate, metabolite production was monitored by HPLC and UV/Vis spectrometry according to a protocol described previously (38).

Docking Studies

Molecular docking of substrates was accomplished using the Schrödinger, Maestro™ suite; version-9.1 (Glide™, version 2.6, Schrodinger, Inc., New York) (39). The crystal structures of BphAE_{LB400} (PDB ID: 2XRX), BphAE_{B9}, and BphAE_{B356} (PDB ID: 3GZX) with bound biphenyl were prepared for docking using Maestro’s protein preparation wizard: hydrogens were added, bond orders were assigned, and the structure was energy minimized using the OPLS2001 force-field until the rmsd
between the minimized structure and the starting structure reached 0.3Å. For each of the prepared protein structures, a 12x12x12 Å receptor grid box was erected using the centroid of the atoms in the bound biphenyl as the center of the box. The substrates, DDT, 2,3,4’-trichlorobiphenyl and 2,2’,5,5’-tetrachlorobiphenyl, were prepared using Maestro’s Ligprep module (Ligprep, Schrodinger, Inc., New York, NY). Glide™ was then used for docking of the substrates using the Extra Precision (XP) mode (40, 41). The best conformation was selected on the basis of the Glide score and Emodel value. Visual inspection was used to confirm that the substrates were docked in a plausible orientation similar to that observed in the crystal structure of the BphAE_{LB400}:biphenyl complex.

Results and Discussion

Crystal structures of BphAE_{19} substrate-free and biphenyl-bound forms

The crystal structures of the substrate-free and biphenyl-bound forms of BphAE_{19} were determined to resolutions of 2.5 Å and 1.9 Å, respectively. Representative crystallographic data and refinement statistics are presented in Table 1.

Coordinates and structure factors for both structures were deposited in the Protein Data Bank with the identifiers 5AEU (substrate-free) and 5AEW (biphenyl bound).

The crystal structure of the substrate-free form was refined at a resolution of 2.5 Å to final $R_{\text{cryst}}$ and $R_{\text{free}}$ values of 18.1% and 23.4%, respectively, with four αβ heterodimers in the asymmetric unit of space group $H3$. Three dimers form one biological hexamer ($\alpha_3\beta_3$) associated with the asymmetric unit, and the fourth forms a hexamer with symmetry related heterodimers. The final model includes residues 18 to 143 and 153 to 459 for all α-subunits and residues 6 to 188 for all β-subunits.

Interpretable electron density was not observed for residues 144 to 152 of the α-subunit. These residues
were also absent in the wild type BphAE_{LB400} structure (12). Similar to other Rieske-type
dioxygenases, each α-subunit binds a [2Fe-2S] Rieske-type cluster and a mononuclear Fe^{2+} ion at the
active site, which is coordinated to two His residues (His233 and His239), an Asp residue (Asp388),
and one or more water molecules.

The biphenyl bound structure was refined to a final $R_{\text{cryst}}$ of 21.6% and $R_{\text{free}}$ of 25.4%, respectively, at a
resolution of 1.9 Å in space group $P1$. The structure includes twelve crystallographically independent
αβ heterodimers. α-subunits are labeled chains A, C, E, G, etc., and β subunits are chains B, D, F, H,
etc. The final model includes residues 18 to 143 and 153 to 459 for all α-subunits except chain U
(missing residues 143 and 459) and chain W (missing residue 143). For the β-subunits, all chains
extend to residue 188, but the N-terminal content varies among the chains: chain F begins at residue 5,
chain J begins at residue 14, and the remaining chains begin within the range of residues 6 to 13.

In eight of the α-subunits (C, E, I, K, M, O, Q, and W), initial unbiased ($F_o-F_c$) Fourier maps showed
bulky electron density at the active site consistent with bound biphenyl, Fig 1(B), whereas such density
was not observed for the other four α-subunits (chains A, G, S, and U). Density consistent with a
partially occupied biphenyl was observed for chain S at a later stage.

Absence of biphenyl in some active sites appears to be a consequence of crystal packing contacts
associated with structural variation in the N-terminal segments of the β subunits. For most β subunits,
the N-terminal segment interacts with residues of the same subunit or an adjacent β subunit of the same
$\alpha_3\beta_3$ hexamer. But for three β subunits (D, F, and J), the N-terminal segment extends away from the
rest of the β subunit and interacts with a loop formed by α-subunit residues 247-263 at the mouth of
one of the active sites of a neighboring $\alpha_3\beta_3$ hexamer. This contact apparently interferes with binding
of biphenyl. In fact, for chains D and F, the side chain of β-Phe9 extends just into the mouth of the
active site of a neighboring α-subunit (chains A and G, respectively) and would prevent entry of 197 substrate into the active site, as shown in Supplementary Fig 1. In the case of chain J, β-Phe9 was not modeled because interpretable electron density begins at residue 14. Nevertheless, the N-terminal segment in chain J extends towards chain U and density for biphenyl was not observed in the active site of U.

Interactions, conformation, and orientation of biphenyl

As observed in prior structures of biphenyl dioxygenases, the substrate binding pocket of BphAE II9 is sandwiched between the core β-strands and α-helices of the α-subunit. Biphenyl binds at the active site in a non-planar conformation with the ring closer to the Fe²⁺ surrounded by residues Gln226, Phe227, Asp230, Met231, His233, and His323. The distal ring is surrounded by Ala234, His239, Ser283, Val287, Gly321, Gln322, Leu333, Ile336, Phe378, and Phe384, Fig 1(B). Non-planarity manifests through torsion about the C1-C1P bond, which averages -52° and ranges from -37° to -67°.

Consistent with a 2,3-dioxygenation of the substrate, the C2 and C3 atoms of biphenyl are generally closer to the Fe²⁺ ion than is C1: the average distances are 4.5 Å for C2 and C3, and 5.1 Å for C1. Despite variations in the C2-Fe²⁺ and C3-Fe²⁺ distances in the range 4.2 Å to 4.8 Å, in no case is either C2 or C3 significantly closer to Fe²⁺. Further, superposition of the α-subunits on the basis of Cα atoms (as described below) places the proximal rings in a tight cluster, as shown in Fig 2(B). The same superposition shows greater variability in the relative positions of the distal rings. This variance in the position of the distal ring is also seen in the structure of biphenyl bound BphAE LB400. However, the proximal ring binds in a similar fashion with respect to the Fe²⁺ ion in all chains.

Water molecules in contact with biphenyl and bound to Fe²⁺ were modeled in three different locations among the several active sites, and either one or two waters were included. Two of the locations
correspond to the site of side-on binding of dioxygen observed in crystal structures of naphthalene dioxygenase (42), whereas the third site lies on the opposite side of a plane defined by C2, C3, and Fe$^{2+}$. Based on the quality of the active site density and the reliability of the atomic coordinates (judged by B factors), the active site models for chains C and O are the most reliable.

Comparison among α-subunits of the substrate-free and biphenyl-bound structures of BphAE$_{119}$

The Cα atoms of the α-subunits from the substrate-free and biphenyl-bound BphAE$_{119}$ structures, 16 chains in total, can be superposed with a Cα rmsd of 0.5 Å or less, demonstrating close agreement. In some regions, however, variations in the local structure are observed.

Examination of superposed αβ dimers reveals that the BphAE$_{119}$:biphenyl complex shows greater variability in the position of key active site elements as shown in Fig 2(B) than either the substrate-free BphAE$_{119}$ structure, Fig 2(C), or the biphenyl-bound form of its progenitor, BphAE$_{EL400}$, Fig 2(A). For example, in the latter two crystal structures, the superposed positions of the Fe$^{2+}$ ions lie in single, tight clusters. By contrast, in the BphAE$_{119}$:biphenyl complex, five Fe$^{2+}$ ions (chains A, C, E, G, and U) are in one cluster and six (I, K, M, O, Q, and W) are found in a second cluster ~1.3 Å distant (average of all intercluster Fe$^{2+}$-Fe$^{2+}$ distances). Although the first cluster includes all chains for which biphenyl is not modeled (A, G, and U), two active sites with biphenyl present (C, E) are also members. The Fe$^{2+}$ from chain S lies approximately between the two clusters and nearly equidistant (0.8 Å) from both.

Comparatively greater positional variability is also observed in the BphAE$_{119}$:biphenyl complex for two of the Fe$^{2+}$ coordinating residues, Asp388 and His239, as well as Ser283, Val287, Gly321, and Gln322. The case of Asp388 is highlighted in Fig 2(A, B, and C). Although the carboxylate groups of Asp388 for all chains lie close to a common plane, the Cδ atoms are as much as 1.5 Å apart. The variations for Asp388 extend to the backbone atoms: the average (0.8 Å) and maximum (1.5 Å) distances among Cα
atoms are indistinguishable from the average and maximum for Ca and Oδ1, which binds to the Fe2+. Ligands would be expected to track with the Fe2+, and thus clusters are again observed for the Asp388 carboxylates, although they are not as distinct or as tightly clustered.

To understand the variations in Fe2+ and Asp388 locations it is useful to consider them with reference to a vector from the Ca atom of Gly321 to the Ca of Asp388, which passes close to the center of biphenyl and close to the Fe2+. The length of the vector is significantly shorter for chains without density for biphenyl, (r = 15.0 Å, range = 15.0 Å to 15.1 Å) compared to chains with biphenyl (r = 16.5 Å, range = 15.9 Å to 17.3 Å). Qualitatively, expansion of the active site along this vector moves the Fe2+ and Asp388 away from Gly321 opening space for biphenyl.

The orientation of the peptide plane between Gly321 and Gln322 and the interactions of the carbonyl of Gly321 also differ between the substrate-free and biphenyl complex and vary among the chains of the latter, Fig 3(A). In all four chains of the BphAEII9 substrate-free structure, ψ is in the range 12° to 37° such that the carbonyl of Gly321 is points into the active site and interacts with Cε of Met231 through a distance of 3.0 Å to 3.2 Å. For the biphenyl soaked structure, the carbonyl of Gly321 is similarly placed in six of the chains including all three without density for biphenyl (A, G, and U) and three with biphenyl modeled (M, Q, and W). In the other six chains, all with biphenyl modeled (C, E, I, K, O, and S), ψ is in the range 95° to 115°, such that the direction of the carbonyl of Gly321 differs by ~90°. This allows the carbonyl to form a hydrogen bond with the amide NH of Tyr277 through a distance of 3.0 Å to 3.1 Å. As in the case of the Fe2+ location, the local structures of chains without biphenyl modeled are consistent, but the chains with biphenyl bound disperse between two groups, one that is consistent with the substrate-free structure and one that is distinct.
A difference in extent of variability between the biphenyl complex and the substrate-free enzyme is also seen for the short α-helix (Pro281-Met288) that forms one side of the active site portal and one wall of the biphenyl binding site, where the side chains of Ser283 and Val287 are in contact with the distal ring of biphenyl. In the BphAE_{II9} biphenyl complex, the maximum difference in the positions of Ser283 Cα atoms for any pair of chains is 1.9 Å, but among the chains in the BphAE_{II9} substrate-free structure the maximum value of the same measure is 0.6 Å.

Finally, a comparable extent of variability is seen in both structures for the backbone and side chain atoms from residue Pro249 to Thr260. Here, the maximum Cα displacement of up to 0.8 Å & 2 Å in BphAE_{II9} biphenyl bound structure and up to 3.8 Å & 1.1 Å is seen in BphAE_{II9} biphenyl free structure at residues Ser254 & Ile258 respectively. A comparable variation is observed for side chain atom positions and conformations. This segment contains a large fraction of solvent exposed residues and does not contribute directly to the biphenyl binding.

**Observed structural changes due to shuffled Region III**

Previous studies suggested that each residue in Region III can influence the functional properties of the enzyme by direct or indirect interactions with active site residues (12, 27). We have compared the structure of BphAE_{II9} with those of the parental enzymes, BphAE_{LB400} and BphAE_{B356}, to consider the influence of specific substitutions on the oxygenase’s functional properties. BphAE_{II9} has four substitutions in Region III with respect to BphAE_{LB400}: Thr335Gly, Phe336Ile, Asn338Thr, and Ile341Thr. In a previous report (29), we showed that the $k_{cat}$ value of BphAE_{II9} and BphAE_{LB400} toward biphenyl is similar. This indicates that the structural changes brought by these substitutions did
not alter the electron transfer system in BphAEII9. However, as we will see below, the structural analysis shows each of these substitutions may contribute to increase flexibility of the active site.

The Thr335Gly substitution: In BphAE_{LB400}, a hydrogen bond between Thr335 Oγ and Gly321 N presumably restricts the conformation of the Val320-Gly321 dipeptide. The Thr335Gly substitution in BphAE_{II9} abolishes the hydrogen bond, and the Val320-Gly321 segment, which lines the active site pocket, is more relaxed, Fig 3(B). As noted above, the 321-322 peptide plane exists in two orientations among the chains of the biphenyl complex. One of these, as illustrated by chain C, would accommodate bulkier substrates. For example, for chain C of BphAE_{II9}:biphenyl complex, a \( \psi \) angle of 107° at Gly321 increases the distance between the carbonyl oxygen of Gly321 and \( \text{ortho} \) and \( \text{meta} \) carbon atoms of biphenyl's distal ring to 5.4 Å and 5.0 Å, respectively. These distances are typically 4 Å or less in the BphAE_{LB400}:biphenyl complex.

As was the case for the previously studied BphAE_{LB400} variant BphAE_{p4}, the substitution of Thr335 by a smaller amino acid such as Ala in BphAE_{p4} or Gly in BphAE_{II9} eliminates a hydrogen bond between the Thr335 Oγ and Gly321 N, relieving a conformational constraint on the Val320-Gly321 dipeptide, residues that line the active site of the protein (12). Due to the absence of this constraint on Gly321, the carbonyl of the peptide bond between Gly321 and Gln322, which points towards the active site in BphAE_{II9} substrate-free structure, points away from the substrate binding site in BphAE_{II9}:biphenyl bound structure. This makes more space in the active site for the binding of bulkier substrates. Also, in the presence of biphenyl, Val320 in BphAE_{II9} is displaced from its original position by 1.0 Å and makes polar contact with Ser283. At its new position, Ser283 interacts with the main chain of residues Ala286 and Val287. This is responsible for the variability in the position of the short \( \alpha \)-helix (Pro281-Met288).
The Phe336Ile substitution: The Phe336Ile substitution places a smaller side chain at the surface of the active site, directly increasing the volume available for substrate binding. The minimum distance between residue 336 and meta and/or para carbons of biphenyl's distal ring increases from 3.6 Å (median) for BphAE_{LB400} (Phe336_{LB400} C\varepsilon and/or C\zeta in a -60,-40 rotamer) to 4.1 Å (median) for BphAE_{II9} (Ile366_{II9} C\delta1 in a -60,-60 rotamer). Moreover, the minimum distance increases to 5.1 Å in chain Q wherein Ile366_{II9} assumes a readily accessible -60,-60 rotamer. It should be noted that mutation to Ile336 affects the conformation of a neighboring residue, Phe378, pushing it closer to biphenyl, Fig 3(C), such that Phe378_{II9} C\zeta approaches the ortho and/or meta atoms of the distal ring. The typical closest contact distances remain longer than 4 Å, but distances as short as 3.7 Å were observed. These contact distances can be readily altered by a change in the torsion angle between biphenyl's rings, whereas contacts between the side chain of residue 336 and the para carbon are unaffected by the conformation of biphenyl.

The Asn338Thr substitution: In this case, an effect mediated by the difference in chain length of Thr and Asn are conceivable. For both variants, the side chain oxygen atom accepts a hydrogen bond from the Ne of neighboring residue, Arg340. It is clearly seen that due to the difference in side chain length of Asn and Thr, the side chain of Arg340 is placed at different positions in BphAE_{LB400} and BphAE_{II9}, respectively, Fig 3(D). While, a hydrogen bond between Arg340 N\eta2 and the carbonyl of Phe378 is clearly visible in BphAE_{LB400} structure, it is not observed in BphAE_{II9}. This impedes the movement of the side chain of Phe378 in BphAE_{LB400} but not in BphAE_{II9}. As mentioned above, Ph378 lies close to the active site and the freedom associated with its side chain can easily affect the orientation of the bound substrate. In BphAE_{II9}, the phenyl ring of Phe378 is 4.9 Å away from the vicinal ring and 4.4 Å away from the distal ring of the biphenyl. These distances in BphAE_{LB400} are 5.5 Å from the vicinal
and 4.9 Å from the distal ring of the substrate. Thus, the type of residue at position 338 indirectly affects the orientation and the position of oxygenation of substrate at the active site.

The Ile341Thr substitution: The influence of this substitution on substrate range or other aspects of enzyme activity remains enigmatic from the perspective of the crystal structures. Relative to the biphenyl binding site, the side chain for position 341 lies on the opposite surface of the central β sheet and its Cβ atom is more than 14 Å distant from the closest atom of biphenyl. Significant changes in local backbone conformation are not observed, and minor changes in the placement or conformation of neighboring side chains directed toward the active site (Arg340 and Trp342) cannot be attributed uniquely to the Ile341Thr substitution.

Therefore, except for the Ile341Thr substitution, structural analysis clearly shows the other three substitutions in BphAE19 may contribute to the expanded substrate range of the enzyme. The indirect effect of Phe336Ile, Asn338Thr, and Ile341Thr substitutions on the active site influences the constraints placed on Phe378, a residue that lines the active site and is particularly close to the bound biphenyl, Fig 4. The Asn338Thr and Ile341Thr substitutions appear to act together pulling the Arg340 side chain away from the backbone carbonyl of Phe378. As a consequence the hydrogen bond involving Arg340 and Phe378 of BphAE_{LB400} is eliminated in BphAE_{19} freeing at least one major constraint on the position of Phe378. In contrast the Phe336Ile substitution acts more directly on the position of Phe378, by displacing the ring of Phe378 in the direction of the Fe^{2+}. Interestingly, by comparing the position of Phe378_{LB400} to the corresponding Phe376_{B356}, Phe376_{B356} exhibits a shift towards the Fe^{2+} as well, Fig. 4.

Thus, although the chains modeled in the structures of BphAE_{19} substrate-free and biphenyl-bound forms show a high degree of similarity with each other and with the chains of BphAE_{LB400}, there are a number of conformational variations within localized regions. The regions of high variability
(specifically the iron ligands Asp388 and His239, the residues Gly321 and Gln322, the α-helix Pro281-His288, and the region of Pro249 through Thr260) demonstrate a high degree of flexibility in and around the active site, Fig 4. Some of this flexibility, for example the Pro249-Thr260 region, is observed in the parent enzyme, but much of it appears unique to BphAE_{LB400}.

Additionally, variation was seen in the binding position and orientation of biphenyl in various αβ-heterodimers of the BphAE_{LB400} biphenyl-bound structure. First, the variation in distance from the Fe^{2+} to the vicinal ring of biphenyl demonstrates a structure with a high tolerance for a variety of substrate binding. Next, the variation seen in the position of the distal ring of biphenyl demonstrates a binding pocket that has a volume larger than is necessary to bind biphenyl, and is capable of binding larger substrates. These aspects of substrate binding again point to a relaxed active site capable of accommodating a variety of substrate analogs which explains the ability of BphAE_{LB400} to metabolize an expanded range of PCB congeners.

Thus, although the chains modeled in the structures of BphAE_{LB400} substrate-free and biphenyl-bound forms show a high degree of similarity with each other and with the chains of BphAE_{LB400}, there are a number of conformational variations within localized regions. The regions of high variability (specifically the iron ligands Asp388 and His239, the residues Gly321 and Gln322, the α-helix Pro281-Met288, and the region of Pro249 through Thr260) demonstrate a high degree of flexibility in and around the active site, Fig 4. Some of this flexibility, for example the Pro249-Thr260 region, is observed in the parent enzyme, but much of it appears unique to BphAE_{LB400}.

Transformation of 2,3,4\textquotesingle-trichlorobiphenyl, 2,2\textquotesingle,5,5\textquotesingle-tetrachlorobiphenyl and DDT and Docking studies

To provide more insight about the structural features in BphAE_{LB400}, BphAE_{LB400}, and BphAE_{LB356} responsible for their differential ability to metabolize biphenyl analogs, we examined the biochemistry...
and the structural interaction of these enzymes toward 2,2',5,5'-tetrachlorobiphenyl, 2,3,4'-trichlorobiphenyl and DDT.

Biochemical studies were performed to assess the ability of BphAE_{II9} to transform DDT and two PCB congeners, 2,3,4'-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl, and the regiospecificity of the reactions.

BphAELB_{400} generated one major metabolite from 2,3,4'-trichlorobiphenyl as determined by examination of the GC-MS profile of the assay solution, Fig 5(A). The mass spectral features of this metabolite’s nBuB derivative were consistent with those of a dihydro-dihydroxy-trichlorobiphenyl, namely a fragmentation pattern exhibiting a molecular ion at m/z 356 and diagnostically important ions at m/z 340 (M⁺ - O), 321 (M⁺ - Cl), 286 (M⁺ - 2Cl), 256 (M⁺ - nBuBO2). A second metabolite exhibiting mass spectral features of a dihydro-dihydroxy-trichlorobiphenyl was also detected, but those features are not observable in Fig 5(A) as this metabolite was only present in trace quantities. BphAE_{II9} transformed 2,3,4'-trichlorobiphenyl into the same major metabolite as shown in Fig 5(A), however, the amount produced was significantly greater. BphAE_{B356} produced the same metabolite from 2,3,4'-trichlorobiphenyl, and the amount produced was similar to BphAE_{LB400} (not shown). Therefore, our assay demonstrated that the major product produced by three enzymes is the same metabolite, and that BphAE_{II9} has a greater ability to degrade 2,3,4'-trichlorobiphenyl as demonstrated by the much larger peak for the metabolite observed in the chromatogram. The substrate was docked into the active site of the three enzymes.

For BphAE_{LB400}, 2,3,4'-trichlorobiphenyl docked with the C3 and C3-Chlorine atoms in approximately the same location as the C2 and C4 atoms of biphenyl in its complex with BphAE_{LB400}, and the proximal ring is in approximately the same plane as found in the biphenyl complex. This places the
In this pose as shown in Supplementary Fig 2(B), C4 and C5 are 4.3 Å and 4.4 Å from the Fe$^{2+}$ ion, respectively, and 2.8 Å and 3.0 Å from a water molecule that occupies the presumed binding site for dioxygen.

For BphAE$_{19}$, docking of 2,3,4'-trichlorobiphenyl produced a pose consistent with the same regio- and stereo-specificity as BphAE$_{LB400}$, but the binding mode is remarkably different, Fig 6(A and B) & Supplementary Fig 2(B). The dichlorinated ring does not penetrate as deeply into the active site, such that its location is comparable to that of the distal ring in the BphAE$_{19}$:biphenyl complex. In addition, relative to the biphenyl complex, the orientation of the dichlorinated ring is shifted by ~70°, and the torsion angle between the rings is shifted by +110°. Nevertheless, the distances from C4 and C5 to the water are 3.3 Å and 3.0 Å, and distances to the Fe$^{2+}$ are 5.1 Å and 4.5 Å. For BphAE$_{LB356}$, 2,3,4'-trichlorobiphenyl docked in a manner similar to BphAE$_{19}$ as shown in Supplementary Fig 2(A), but with reduced docking scores.

Our docking study showed that the preferred docking pose for all three enzymes was one that positioned the substrate to be converted to a 3,4-dihydro-3,4-dihydroxy-2,3,4'-trichlorobiphenyl. This proposed product would not be further degraded into a chlorobenzoate, which may explain why in their experiment with BphAE$_{LB400}$, Seeger et al. did not detect any chlorobenzoate produced from 2,3,4'-trichlorobiphenyl since BphAE$_{LB400}$ metabolize this substrate through a 3,4-dioxygenation (43).

Further, the docking study showed that the preferred pose for BphAE$_{LB400}$ oriented the substrate much further into the active site than the preferred pose for BphAE$_{19}$, Fig 6(B) and Supplementary Fig 2(B). This deeper binding mode may explain why BphAE$_{LB400}$ exhibits a lower activity towards 2,3,4'-trichlorobiphenyl than BphAE$_{19}$. 
BphAE_{119} and BphAE_{LB400} produced dihydro-dihydroxy-tetrachlorinated as the only metabolite of 2,2',5,5'-tetrachlorobiphenyl and on the basis of peak area, the amounts produced were approximately the same for both enzymes, Fig 5(B). Consistent with a previous report (44), the dihydro-dihydroxy-tetrachlorobiphenyl produced from 2,2',5,5'-tetrachlorobiphenyl by BphAE_{119} and BphAE_{LB400} must have been the 3,4-dihydro-3,4-dihydroxy-2,2',5,5'-tetrachlorobiphenyl and GC-MS analysis showed that the metabolite produced by BphAE_{119} was the same as the one produced from BphAE_{LB400}.

The bulkier 2,2',5,5'-tetrachlorobiphenyl docked at the active sites of BphAE_{LB400}, BphAE_{119}, and BphAE_{B356} in distinct locations and orientations, Fig 7(B). Once again, the deepest penetration was found for BphAE_{LB400}, where docking of 2,2',5,5'-tetrachlorobiphenyl gave a single pose competent for dioxygenation at C3 and C4, consistent with available biochemical data (Table 2). C3 and C4 are almost equidistant from the key water molecule at 3.3 Å, and from the Fe^{2+} ion at 4.2 Å, Supplementary Fig 3(B). The C5-chlorine faces towards His323, the C2-chlorine points towards Phe384, and the proximal ring is nearly in the same as observed for the proximal ring of biphenyl in its complex with BphAE_{LB400}.

In the active site of BphAE_{119}, 2,2',5,5'-tetrachlorobiphenyl docked in multiple poses that would allow ortho-meta dioxygenation across the C5-C6 or C2-C3 bonds as well as meta-para dioxygenation across the C3-C4 bond. Since dechlorinated products were not observed in the biochemical analysis, poses consistent with ortho-meta dioxygenation were ruled out for the purposes of this study. The orientation most competent for meta-para dioxygenation, consistent with the biochemical data, was chosen for further analysis although its docking metrics were lower. In this pose as shown in Fig 7(A), carbon 3 and 4 are 3.0 Å and 4.2 Å away from the water molecule and 4.1 Å and 5.4 Å away from the Fe^{2+} ion.

Unlike BphAE_{LB400}, the chlorine at the 2-C position of 2,2',5,5'-tetrachlorobiphenyl is positioned...
towards His239 in BphAE\textsubscript{II9}. The chlorine at the 5-C position faces toward His323 BphAE\textsubscript{II9}, but in a
different orientation than in BphAE\textsubscript{LB400}.

Interestingly, the docking experiments of the two enzymes showed very different preferred poses for
the binding of this substrate. The binding pose of 2,2',5,5'-tetrachlorobiphenyl in BphAE\textsubscript{II9} would
likely generate a steric conflict between the substrate and Phe336 if adopted in BphAE\textsubscript{LB400}. The pose
adopted in BphAE\textsubscript{LB400}, likewise, appears to be inaccessible to BphAE\textsubscript{II9}, as it would create a steric
conflict with Phe378. But as discussed above Phe378 is less constrained in BphAE\textsubscript{II9} than in
BphAE\textsubscript{LB400}. Therefore, Phe378\textsubscript{II9} may be more flexible in reality than was allowed in our docking
experiments which may explain the differences between the biochemical and the docking experiments.

For BphAE\textsubscript{B356}, the best scoring pose places 2,2',5,5'-tetrachlorobiphenyl much further from the water
(4.1 Å and 4.3 Å) and Fe\textsuperscript{2+} (5.6 Å to both carbons) as compared to BphAE\textsubscript{LB400} and BphAE\textsubscript{II9},
Supplementary Fig 3(A) and Table 2. C3 and C4 are closest to the Fe\textsuperscript{2+}, but the increased distances
reflect a binding mode that is likely incompetent for the reaction, consistent with biochemical data that
show BphAE\textsubscript{B356} has poor activity towards 2,2',5,5'-tetrachlorobiphenyl.

BphAE\textsubscript{II9} retains activity towards 2,2',5,5'-tetrachlorobiphenyl while BphAE\textsubscript{B356} shows very little
activity towards that particular congener. This apparent contradiction is resolved when you consider the
role of an adjacent residue, Thr375\textsubscript{B356}, on the position of Phe376\textsubscript{B356}. Thr375\textsubscript{B356} Oγ1 makes a
hydrogen bond to the backbone carbonyl of Glu371\textsubscript{B356}. This hydrogen bond forces Phe376\textsubscript{B356} to a
position closer to the Fe\textsuperscript{2+}, Fig 8. The corresponding residue to Thr375\textsubscript{B356} is Asn377\textsubscript{LB400/II9}.
Asn377\textsubscript{LB400/II9} also forms hydrogen bond to the backbone carbonyl of His373\textsubscript{LB400/II9} (corresponding to
Glu371\textsubscript{B356}) and with the backbone carbonyl of Val287\textsubscript{LB400/II9}, Fig 8. This second interaction is
missing in BphAE\textsubscript{B356} and appears to anchor Phe378\textsubscript{II9} and restricts how far towards the Fe\textsuperscript{2+} the
residue can move, and thus seems responsible for preserving BphAE$_{II9}$’s activity towards 2,2’,5,5’-tetrachlorobiphenyl.

Based on this analysis one would expect that if Thr$_{375}^{B356}$ was mutated to an Asn, BphAE$_{B356}$ could potentially gain the ability to oxygenate 2,2’,5,5’-tetrachlorobiphenyl. Indeed, BphAE$_{KF707}$, a strain that exhibits no activity towards 2,2’,5,5’-tetrachlorobiphenyl, gains the ability to oxygenate that particular congener by a Thr$_{376}^{Asn}$ substitution (corresponding to Thr$_{375}^{B356}$ and Asn$_{377}^{LB400/II9}$) (45, 46). Likewise, the reverse is true, when BphAE$_{LB400}$’s Region III is replaced by Region III of BphAE$_{KF707}$ along with mutating Asn$_{377}^{LB400}$ to Thr (as in BphAE$_{KF707/B356}$) the enzyme loses its activity against ortho-substituted congeners (47). However, it was also reported by Mondello et al. that the Asn$_{377}^{LB400}$Thr substitution alone (i.e. without swapping of Region III) did not affect the substrate specificity of the mutated enzyme (24). Thus, neither the single Asn$_{377}$Thr substitution, nor the swapping of Region III alone appear to restructure BphAE$_{LB400}$ enough to disrupt its ability to act against ortho-substituted congeners.

As reported previously (38), on the basis of product formation, the $K_m$ and $k_{cat}$ values of BphAE$_{B356}$ toward DDT were 174 ± 5 µM and 0.15 ± 0.08 s$^{-1}$, respectively. During the current work we found that the activity of BphAE$_{LB400}$ towards DDT was too poor to calculate accurate steady-state kinetic values. On the other hand, BphAE$_{II9}$ was able to metabolize DDT, exhibiting $K_m$ and $k_{cat}$ values of 82 ± 2 µM and 0.18 ± 0.04 s$^{-1}$, respectively. The GC-MS chromatogram produced from BphAE$_{B356}$ against DDT shows two metabolites identified as stereoisomers of 1,1,1-trichloro-2,(4-chlorophenyl-2,3-dihydroxy-4,6-cyclohexadiene)-2-(4’-chlorophenyl)ethane as shown in Fig 5(C). BphAE$_{II9}$ produced the same two stereoisomers but in inverse ratio to that of BphAE$_{B356}$, Fig 5(C).
The active sites of BphAE_{II9}, BphAE_{LB400}, and BphAE_{B356} all accommodated DDT with corresponding 4-Cl-phenyl rings in similar locations. For the proximal ring, the distances between C4 atoms are 1.1 Å - 1.4 Å, and the orientations of the rings are also similar, Fig 9 (B). Nevertheless, the placement of the trichloromethyl group distinguishes the BphAE_{LB400} complex from the others, and this complex had the poorest docking metrics (Table 2).

For BphAE_{II9}, the top-ranked pose is consistent with dioxygenation across the C2-C3 bond, and the trichloromethyl group is directed away from the Fe^{2+} ion toward Gly321, Fig 9(A). The distances from C2 and C3 of the proximal ring to the water that marks the dioxygen binding site are 2.6 Å and 2.3 Å, and the distances to Fe^{2+} are 4.5 Å and 4.1 Å. The best pose for BphAE_{B356} is similar, Supplementary Fig 4(A), but the C2 and C3 atoms are more distant from the water atom, 3.3 Å and 3.1 Å, and the Fe^{2+} ion, 4.9 Å and 4.3 Å. The trichloromethyl group points in a similar direction toward Gly319 (aligns with Gly321 of BphAE_{II9}).

In the best pose for the BphAE_{LB400} complex, the C2 and C3 atoms lie 3.4 Å from the water and 4.0 Å and 4.1 Å from the Fe^{2+}, however, the trichloromethyl group lies near Phe378 and the Fe^{2+} ion as shown in Supplementary Fig 4(B) such that the shortest Cl-Fe^{2+} distance is 4.1 Å. Moreover, although the C-water and C-Fe^{2+} distances are similar in the three docked complexes, the geometric relationships between the ring, the water, and the Fe^{2+} in BphAE_{LB400} differ markedly. In the BphAE_{II9} and BphAE_{B356} complexes, the plane defined by C2, C3, and the Fe^{2+} is nearly orthogonal to the plane of the ring, and the water is only ~0.8 Å out of the C2-C3-Fe^{2+} plane but more than 2.0 Å out of the plane of the ring. By contrast, in the BphAE_{LB400} complex, the angle between the planes is ~45° and the water is ~1.5 Å away from the the C2-C3-Fe^{2+} plane and within 1.0 Å of the plane of the ring.
Therefore, our study demonstrates that BphAE_{II9} and BphAE_{B356} have a similar level of activity towards DDT. This finding is further supported by the docking study that shows BphAE_{B356} and BphAE_{II9} have preferred binding poses for DDT that were quite similar. Unlike the other two enzymes, BphAE_{LB400} metabolized DDT very poorly and the docking experiments generated a binding pose that was completely different from that of the other two enzymes. On the basis of biochemical data, the preferred binding pose modeled in BphAE_{LB400} is not a productive orientation. However, if DDT was oriented in BphAE_{LB400} in a way similar to what was observed in the docking with BphAE_{II9}, the substrate would interfere with Gly321-Gln322 and Phe336. This interference is not present in BphAE_{II9} due to the substitution of Phe336 with Ile and the elimination of a hydrogen bond that constrains the position of Gly321-Gln322.

In conclusion, our study of the structure of BphAE_{II9} suggests an enzyme with increased flexibility in and around its active site when compared to its two parent enzymes. This increased flexibility would allow it to better accommodate a wide variety of potential substrates and this is reflected in its enhanced substrate profile compared to its parent enzymes.

Acknowledgement

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Figure legend:

Fig 1: A) Schematic representation of the first enzyme of biphenyl catabolic pathway exhibiting its three components. B) Displaying the bound biphenyl in BphAE19 with Fo - Fc electron density.
contoured at 3σ. Residues surrounding the biphenyl are shown in stick models. The Fe$^{2+}$ atom and water molecule are shown as spheres.

**Fig 2:** Superposition of all the chains of A) BphAE$_{LB400}$ biphenyl bound, B) BphAE$_{I99}$ biphenyl bound C) BphAE$_{I99}$ substrate free structure with Fe$^{2+}$ and conserved water atom has been shown exhibiting the differences in the conformation of the residues at the active site. For distinction, five Fe$^{2+}$ ions in cluster I (chains A, C, E, G and U) are colored orange and in cluster II (chains I, K, M, O, Q and W) are colored blue. In chain S, Fe$^{2+}$ is colored yellow. Water molecules bound at the active site are colored red. The red box accentuates the alterations in Asp388 residue in BphAE$_{I99}$ biphenyl bound structure in comparison with other structures.

**Fig 3:** Stereo view showing the active site region of BphAE$_{LB400}$ (blue), BphAE$_{B356}$ (red), BphAE$_{I99}$-biphenyl bound (green), BphAE$_{I99}$-biphenyl free (magenta) superposed over each other. (A) The variation in the position of carbonyl towards the active site in BphAE$_{LB400}$, in BphAE$_{I99}$-biphenyl free structure and away from the active site in BphAE$_{I99}$ due to mutation in residue Thr335Gly has been shown. (B) The effect of residue Thr at position 335 in BphAE$_{LB400}$ and the effect of its mutation to Gly in BphAE$_{I99}$ has been shown. B) The effect of residue Phe at position 336 in BphAE$_{LB400}$ and the effect of its mutation to Ile in BphAE$_{I99}$ has been shown. C) The effect of residue Asn at position 338 in BphAE$_{LB400}$ and the effect of its mutation to Thr in BphAE$_{I99}$ has been shown. For ease, Fe$^{2+}$ has been colored with the corresponding color of carbon in each structure. The dotted line in yellow has been used to show the distances measured in Å. The other dotted lines in blue, red and green highlights hydrogen bond in BphAE$_{LB400}$, BphAE$_{B356}$ and BphAE$_{I99}$-biphenyl bound with surrounding residues respectively.
Fig 4: Figure showing the overall differences at the active site of BphAE<sub>II9</sub> (in cyan color) due to mutation in comparison with the ligand bound state of BphAE<sub>LB400</sub> (in green color). The red arrows show the effect of residues at position 336, 338, and Arg340 on Phe378. The boxes in red color show other important regions where large differences have been found between BphAE<sub>LB400</sub> and BphAE<sub>B356</sub>. For ease, the Fe<sup>2+</sup> ion has been colored with the corresponding color of carbon in each structure. The dotted line in yellow has been used to show the distances measured in Å.

Fig 5: Total ion chromatogram showing A) the peak of the metabolite produced from 2,3,4′-trichlorobiphenyl and the peak of the substrate remaining after 15 min reaction by reconstituted His-tagged BphAE<sub>II9</sub> (black curve), BphAE<sub>LB400</sub> (gray curve), B) the peak of the metabolite produced from 2,2′,5,5′-tetrachlorobiphenyl and the peak of the substrate remaining after 15 min reaction by reconstituted His-tagged BphAE<sub>II9</sub> (black curve), BphAE<sub>LB400</sub> (gray curve), C) the peaks of the two stereoisomer metabolites produced from DDT after 15 min reaction by reconstituted His-tagged BphAE<sub>II9</sub> (grey curve), BphAE<sub>B356</sub> (black curve).

Fig 6: Stereo view of structure of (A) BphAE<sub>II9</sub> & (B) superposed structures of BphAE<sub>LB400</sub> (red), BphAE<sub>B356</sub> (blue), and BphAE<sub>II9</sub> (yellow-orange) at the active site in the presence of Fe<sup>2+</sup> and water molecule with the surrounding residues with docked 2,3,4′-trichlorobiphenyl in a pose which has shown maximum biochemical output, i.e., 4,5-dioxygenation. The color mentioned above corresponds to the color of carbon in the figure. Chlorine atoms are green, oxygen atoms are red, and nitrogen atoms are blue. For ease, the Fe<sup>2+</sup> ion and water have been colored with the corresponding color of carbon in each structure. The sphere with the larger radius corresponds to Fe<sup>2+</sup> and the other to the water. The dashed lines are the calculated distances measured in Å from docked substrate and Fe<sup>2+</sup> or water molecule.
**Fig 7:** Stereo view of structure of (A) BphAE<sub>II9</sub> & (B) superposed structures of BphAE<sub>LB400</sub> (red), BphAE<sub>B356</sub> (blue), and BphAE<sub>II9</sub> (yellow-orange) at the active site in the presence of Fe<sup>2+</sup> and water molecule with the surrounding residues with docked 2,5,2',5'-tetrachlorobiphenyl in a pose which has shown maximum biochemical output, *i.e.*, 3,4-dioxygenation. The color mentioned above corresponds to the color of carbon in the figure. Chlorine atoms are green, oxygen atoms are red, and nitrogen atoms are blue. For ease, the Fe<sup>2+</sup> ion and water have been colored with the corresponding color of carbon in each structure. The sphere with the larger radius corresponds to Fe<sup>2+</sup> and the other to the water. The dashed lines are the calculated distances measured in Å from docked substrate and Fe<sup>2+</sup> or water molecule.

**Fig 8:** Stereo view of structure of (A) BphAE<sub>II9</sub> & (B) superposed structures of BphAE<sub>LB400</sub> (red), BphAE<sub>B356</sub> (blue), and BphAE<sub>II9</sub> (yellow-orange) at the active site in the presence of Fe<sup>2+</sup> and water molecule with the surrounding residues with docked 2,5,2',5'-tetrachlorobiphenyl showing the effect of Thr375 in BphAE<sub>B356</sub> and corresponding residue Asn377 in BphAE<sub>LB400/BII9</sub> is shown. For ease, the Fe<sup>2+</sup> ion and water have been colored with the corresponding color of carbon in each structure. The sphere with the larger radius corresponds to Fe<sup>2+</sup> and the other to the water. The blue, red and yellow-orange dotted line shows the H-bond interactions of different residues in BphAE<sub>B356</sub>, BphAE<sub>LB400</sub> and BphAE<sub>II9</sub>.

**Fig 9:** Stereo view of structure of (A) BphAE<sub>II9</sub> & (B) superposed structures of BphAE<sub>LB400</sub> (red), BphAE<sub>B356</sub> (blue), and BphAE<sub>II9</sub> (yellow-orange) at the active site in the presence of Fe<sup>2+</sup> and water molecule with the surrounding residues with docked DDT in a pose which has shown maximum biochemical output, *i.e.*, 2,3-dioxygenation. The color mentioned above corresponds to the color of carbon in the figure. Chlorine atoms are green, oxygen atoms are red, and nitrogen atoms are blue. For ease, the Fe<sup>2+</sup> ion and water have been colored with the corresponding color of carbon in each
structure. The sphere with the larger radius corresponds to Fe$^{2+}$ and the other to the water. The dashed lines are the calculated distances measured in Å from docked substrate and Fe$^{2+}$ or water molecule.
Table Title:

**Table1**: Data collection and refinement statistics for biphenyl-free and biphenyl-bound structure of BphAE_{119}.

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## Refinement

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<td>( R_{\text{free}} ) (%)</td>
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### Average \( B \)-factors (Å²)

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<th>GH</th>
<th>IJ</th>
<th>KL</th>
<th>MN</th>
<th>OP</th>
<th>QR</th>
<th>ST</th>
<th>UV</th>
<th>WX</th>
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<th>Water Atoms</th>
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\[ R_{\text{sym}} = \frac{\sum_{hk} \sum_{i,j} |I_{hk,i} - I_{hk,j}|^{2}}{\sum_{hk} \sum_{i} I_{hk,i}^{2}} \]
Table 2: Table showing the probable sites for dioxygenation, the distances from those carbons to water and Fe\(^{2+}\), the Glide score, and the Emodel value for the docking of 2,3,4'-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl and DDT at the active site of BphAE\(_{LB400}\), BphAE\(_{B356}\), and BphAE\(_{II9}\).

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<th>Emodel</th>
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