Rieske-type oxygenases (ROs) catalyze a stereospecific oxygenation of many aromatic and hetero-aromatic molecules. These enzymes have potential applications as biocatalysts to degrade persistent pollutants, such as polyaromatic hydrocarbons (1,2), polychlorinated biphenyls (PCBs) (3-5), chlorodibenzo furans (6,7), or to produce chiral arene cis-dihydrodiols of interest in enantioselective syntheses for the manufacture of fine chemicals (8-10). Biphenyl dioxygenase (BPDO), one of the most extensively studied ROs, catalyzes the first reaction of the bacterial biphenyl catabolic pathway. BPDO has three components: the iron-sulfur oxygenase (hereinafter referred to as BphAE), a heterohexamer comprised of three α (Mr = 51,000) and three β (Mr = 22,000) subunits; the ferredoxin (BphF, Mr = 12,000); and the ferredoxin reductase (BphG, Mr = 43,000). The encoding genes for Burkholderia xenovorans LB400 (11), which is the best PCB degrader of natural origin, are bphA (BphAE LB400 α subunit), bphE (BphAE LB400 β subunit), bphF (BphF LB400) and bphG (BphG LB400) (Fig. 1).

BphAE LB400 has been thoroughly investigated because it can oxygenate a broad range of substrates. BphAE LB400 variants with extended substrate range have been generated by site-directed mutagenesis (12) and directed evolution (13,14). However, the enzyme’s structural features that modulate substrate range and catalytic efficiency have yet to be determined. Many investigations have identified residues in contact
with the substrate, or removed from it, as key determinants of substrate preference and regiospecificity (12-23). Recently Thr$^{335}$ of BphAE$_{LB400}$, which is removed from the substrate, was found to restrain the range of chlorobiphenyls the enzyme can oxidize by controlling the spatial distribution of protein atoms in contact with the substrates (17). Changing Thr$^{335}$ to Ala relieves intramolecular constraints on Gly$^{321}$ allowing for significant movement of this residue during substrate binding, thereby increasing the space available to accommodate the bulkier substrate 2,6-dichlorobiphenyl. In addition, crystal structures of the oxygenase component of carbazole 1,9a-dioxygenase and of the binary complex of the oxygenase and ferredoxin components provided evidence that conformational changes are required to suitably align the Rieske clusters of the ferredoxin and oxygenase components (24). These observations are consistent with a mechanism whereby an induced-fit process is involved in ROs substrate binding and catalytic function. Understanding how conformational adjustments influence the turnover rate and how they can be modified to enhance activity toward new substrates will aid the development of new, better-performing catalysts.

Unlike biphenyl, the fused rings of dibenzofuran are locked in a co-planar conformation (Fig. 1), and this molecule is poorly oxygenated by BphAE$_{LB400}$ (25,26). In previous reports (7,14), we described variant BphAE$_{p4}$ obtained by the double substitution of Thr$^{335}$Phe$^{336}$ of BphAE$_{LB400}$ to Ala$^{335}$Met$^{336}$ and variant BphAERR$_{41}$ obtained by changing Asn$^{338}$Ile$^{341}$Leu$^{343}$Phe of BphAE$_{p4}$ (Table 1). These bphAE mutants were cloned in pQE31 or in pET14b. DNA protocols were generally according to Sambrook et al. (29). DNA from each mutant was sequenced at the Genome Quebec DNA Sequencing Center (Montreal, Quebec, Canada). Biphenyl and dibenzofuran were of the highest purity grade available from AccuStandard (New Haven, CT).

**EXPERIMENTAL PROCEDURES**

**Strains and plasmids-** Escherichia coli DH11S (27) and C41(DE3) (28) (Statagene, La Jolla, CA) were used in this study. The wild-type (WT) BphAE$_{LB400}$ and its mutants BphAE$_{p401}$ (T335A), BphAE$_{p402}$ (F336M), BphAE$_{p4}$ (T335A/F336M) and BphAERR$_{41}$ (T335A/F336M/N383Q/I341V/L409F) were described previously (17).

A previously described two-step site-directed mutagenesis protocol (7) was used to create a set of mutants representing the six mutants that can be produced by single and double substitutions of Asn$^{338}$Gln, Ile$^{341}$Val and Leu$^{343}$Phe of BphAE$_{p4}$ (Table 1). These bphAE mutants were cloned in pQE31 or in pET14b. DNA protocols were generally according to Sambrook et al. (29). DNA from each mutant was sequenced at the Genome Quebec DNA Sequencing Center (Montreal, Quebec, Canada). Biphenyl and dibenzofuran were of the highest purity grade available from AccuStandard (New Haven, CT).

**Protein analysis-** The level of expression of each variant enzyme in IPTG-induced E. coli DH11S pDB31[LB400-bphFG] + pQE31[bphAE] was assessed by SDS-PAGE (30) of crude cell extracts prepared under benign (cells were sonicated in 10 mM phosphate buffer pH7.3 containing 140 mM NaCl) or denaturating conditions (cells were sonicated in 10 mM phosphate buffer pH7.3 containing 140 mM NaCl and 8 M urea). Gels were stained with Coomassie Brilliant Blue. Purified enzyme preparations were also analyzed by HPLC gel filtration chromatography using a Waters Protein Pak 300 SW column (7.8 x 300 mm), as described previously (31).

**Monitoring enzyme activity with purified enzyme preparations-** Reconstituted His-tagged purified BPDO preparations were used to monitor enzyme activity and metabolite production. In this case, the genes expressing each enzyme component were cloned into pET-14b (Novagen, Madison, WI) and expressed in E. coli C41(DE3). The components were produced as recombinant His-tagged protein and purified by affinity chromatography on high performance Ni-
Sepharose resin (GE Healthcare) (7). The concentration of each purified component was determined by spectrophotometry (31-33). Enzymatic reactions were performed as described previously (7) at 37°C, in pH 6.0 MES 50 mM buffer, and in a volume of 400 µl containing 1.2 nmol of each of the His-tagged enzyme component and 200 nmol NADH. Substrate depletion and metabolite production were analyzed and quantified by gas chromatography-mass spectrometry (GC-MS) using previously published protocols (7). The steady-state kinetic parameters of all BphAEs were determined by recording oxygen consumption rates using a Clarke-type Hansatech model DW1 oxygraph (34) for concentrations of biphenyl and dibenzofuran varying between 5 and 150 µM. Kinetic parameters reported in this investigation were obtained from analysis of at least two independently produced preparations tested in triplicate.

Crystal structure analyses- Purification, crystallization, and preliminary X-ray diffraction properties of BphAERR41 have been communicated elsewhere (35). The procedures to prepare crystals of BphAERR41 and its dibenzofuran-bound form were identical to those described for BphAE<sub>p4</sub> (17). The crystal structures were obtained and analysed using the same approaches and software used in studies of BphAE<sub>p4</sub> (17). Crystal structures of BphAERR41 and its dibenzofuran-bound form were compared to crystal structures of BphAE<sub>LB400</sub> (RCSB Protein Data accession codes 2XR8) and its biphenyl-bound form (2XRX) as well as BphAE<sub>pt</sub> (2XSO) and its 2,6-dichlorobiphenyl-bound form (2XSH).

PDB accession codes- The coordinates have been deposited with the RCSB Protein Data Bank (http://deposit.rcsb.org/) under accession codes 2YFI for BphAERR41 and 2YFJ for its complex with dibenzofuran.

RESULTS

Steady-state kinetics of BphAE<sub>pt</sub>, BphAERR41 and their variants with dibenzofuran- Based on the sum of areas under GC-MS peaks of metabolites produced when 1.2 nmol enzyme was incubated for 2 min with 100 µM dibenzofuran, BphAE<sub>pt</sub> and BphAERR41 produced, respectively, three and four times more metabolites than BphAE<sub>LB400</sub> (Fig. 2). Consistent with the single time-point measurements, the apparent k<sub>cat</sub> value for BphAERR41 is approximately 1.5 times higher than that of BphAE<sub>pt</sub> and 3 times higher than for BphAE<sub>LB400</sub> based on the oxygen consumption rates recorded for variable concentrations of dibenzofuran (Table 2). These results show the superior ability to metabolize dibenzofuran of BphAERR41 compared to BphAE<sub>pt</sub> and BphAE<sub>LB400</sub>.

In order to identify the mutations that contribute most to the enhanced activity of BphAE<sub>pt</sub> and BphAERR41 toward dibenzofuran, we assayed all mutants carrying single or multiple mutations at positions 335, 336, 338, 341 or 409 (Table 1). Based on the sum of areas under GC-MS peaks, the amounts of metabolites produced by the T335A mutant and by BphAE<sub>pt</sub> (T335A/F336M) were similar and about three times higher than the amounts for BphAE<sub>LB400</sub> and its F336M mutant (Fig. 2). The superior ability of the T335A mutant to metabolize dibenzofuran was confirmed by steady-state kinetics (Table 2). Furthermore, the single F336M substitution lowered k<sub>cat</sub> and k<sub>cat</sub>/K<sub>m</sub> for the reaction with biphenyl. This identified the T335A substitution as responsible for the enhanced ability of BphAE<sub>pt</sub> to metabolize dibenzofuran.

The apparent k<sub>cat</sub> values for the mutants T335AF336M/I341V and T335AF336M/I341V/L409F toward biphenyl and dibenzofuran were lower than for BphAE<sub>pt</sub>. In addition, introducing the single Ile<sup>341</sup>Val or the double Ile<sup>341</sup>Val Leu<sup>409</sup>Phe mutations into BphAE<sub>pt</sub> did not contribute to enhanced activity toward dibenzofuran. We did not determine steady-state kinetic parameters for variant T335AF336M/L409F. However, the observation that recombinant E. coli cells producing this enzyme did not degrade dibenzofuran more efficiently than BphAE<sub>pt</sub> (not shown) indicates that changing Leu<sup>409</sup> to Phe did not influence activity toward dibenzofuran. Variants T335AF336MN338Q and T335AF336MN338Q/I341V were poorly active toward biphenyl and dibenzofuran because they are not assembled correctly (see below). The
steady-state kinetic parameters obtained with these two variants were too difficult to determine accurately and therefore are not reported here. On the other hand, the apparent $k_{cat}$ value for T335AF336MN338QL409F toward dibenzofuran was in the same range or even higher than for BphAE$_{RR41}$ (T335AF336MN338Q/L341V/L409F) (Table 2). Furthermore, replacing Asn$^{338}$Leu$^{409}$ of BphAE$_{p4}$ with Gln$^{338}$Phe$^{409}$ created a double mutant exhibiting enhanced activity toward dibenzofuran.

The poor activity of variants T335AF336MN338Q and T335AF336MN338Q/I341V is the result of hexamer misassembly. Unlike BphAE$_{RR41}$ and T335AF336MN338Q/L409F, variants T335AF336MN338Q and T335AF336MN338Q/I341V were poorly active, which suggests a detrimental effect of the Asn$^{338}$Gln substitution on enzyme activity. When the proteins of IPTG-induced recombinant E. coli cells producing variant BphAE$_{s}$s were extracted under denaturing conditions and separated by SDS-PAGE, the intensities of bands corresponding to the $\alpha$ and the $\beta$ subunits were similar for all strains (Fig. 3A). This shows the level of expression of BphAE is similar for all IPTG-induced E. coli clones expressing these variants. However, analysis of cell extracts prepared under non-denaturing conditions revealed significantly lower intensities of the bands corresponding to the $\alpha$ subunits for variant T335AF336MN338Q and T335AF336MN338Q/I341V compared to the other variants (Fig. 3B). This suggests misassembly or early dissociation of $\alpha_3\beta_3$ hexamers resulting in loss of $\alpha$ subunits into the non-soluble protein fraction. HPLC gel filtration analysis of freshly purified His-tagged BphAE of variants T335AF336MN338Q and T335AF336MN338Q/I341V confirmed that less than 10% of each protein preparation exhibited the expected $\alpha_3\beta_3$ association pattern (not shown). Therefore, the Asn$^{338}$Gln substitution hinders hexamer assembly unless a concomitant Leu$^{409}$Phe substitution is introduced. Nevertheless, the double Asn$^{338}$Gln Leu$^{409}$Phe substitution is beneficial for enhancing catalytic properties towards dibenzofuran.

**Overall structure of BphAE$_{RR41}$** The overall structure of BphAE$_{RR41}$ and of its dibenzofuran-bound form are very similar to the crystal structures of the BphAE$_{p4}$:2,6-dichlorobiphenyl complex (17), which contains triplets of $\alpha\beta$ dimers associated into two hexamers in the asymmetric unit (chains ABCDEF and chains GHIJKL). For both structures, the final refined models contain residues Asn$^{18}$ to Phe$^{143}$ plus Phe$^{153}$ to Pro$^{459}$ of each $\alpha$ subunit, residues Phe$^{9}$ to Phe$^{188}$ of each $\beta$ subunit and 1217 water molecules. The diffraction data and refined models are characterized in Table 3.

Superposition of all C$^{\alpha}$ atoms for the $\alpha\beta$ dimer of chains AB with chains CD-KL yielded rmsd values of 0.2-0.3 Å. The poorly ordered residues and protein segments were the same as observed for BphAE$_{p4}$ (17), including segments comprising residues Ile$^{247}$ to Lys$^{263}$ and Glu$^{280}$ to Val$^{287}$ of the $\alpha$ subunit and 9-17 and 158-164 of the $\beta$ subunit. In the BphAE$_{RR41}$:dibenzofuran complex structure, dibenzofuran could be identified clearly in ($F_o$-$F_c$) difference Fourier maps in all active sites of the ABCDEF hexamer. Similar to BphAE$_{LB400}$:biphenyl, a water molecule lies between the Fe$^{++}$ and dibenzofuran at approximately 2 Å from the catalytic iron. Electron density maps of the active site residues of the BphAE$_{RR41}$ and of its dibenzofuran-bound form are shown for dimer AB in Fig. 4.

When the BphAE$_{RR41}$:dibenzofuran BphAE$_{LB400}$:biphenyl structures are superposed, the positions of the carbons targeted for hydroxylation, C-4a and C-4 of dibenzofuran and C-2 and C-3 of biphenyl, are nearly the same in $\alpha\beta$ dimers AB, CD and EF (not shown). In $\alpha\beta$ dimers AB, CD and EF of BphAE$_{RR41}$:dibenzofuran, the substrate is in an orientation that would favor 4,4a angular attack. This is unexpected since biochemical data revealed dioxygenation of the lateral 1,2 and 3,4 carbons of dibenzofuran as by far most favored for BphAE$_{RR41}$ (7). In the crystal structure, the furan ring's oxygen atom contacts the water ligand of the active site Fe$^{++}$ atom. Based on observations drawn from the naphthalene dioxygenase (NDO) crystal structure, Karlsson et al. (36) proposed a reaction cycle for ROs in which Fe$^{++}$ coordinates this water in states prior to dioxygen binding. When dioxygen binds it intercalates side-on
between the iron and the substrate displacing the water. Thus, a subsequent adjustment in substrate position or orientation is possible such that the lateral attack would occur. In essence, the dibenzofuran-O-water interaction seen in the crystal form could produce a false inference about the points of attack because the structure reports on a state prior to dioxygen binding.

**Structural analysis of the influence of residue 335 on catalytic properties toward dibenzofuran**

The average active site cavity volume of the αβ dimers AB, CD, EF of BphAERR41 was comparable to that of BphAELB400 (17) (1073 Å³ as calculated using CASTp program (37)). The corresponding atoms of the reactive ring of dibenzofuran and of biphenyl interact with the same residues of the α subunits of BphAERR41 and BphAELB400 (Gln226, Phe227, Asp230, Met231, Leu233, Ala234, His235 and Leu333) and they are located at approximately the same distances (not shown). Therefore, neither the overall size of the cavity nor the constraints on the reactive ring are affected by the mutations introduced in BphAERR41. The residues lining the distal portion of the BphAERR41 catalytic cavity are the same as those in BphAELB400 and BphAELB400 (Phe384, Phe378, Val1287, Ser283, Phe/Met336, Leu333, Gly321, Tyr277, His239, Ala234, and Met231) (Fig. 5). However, the overall shape of the cavity of BphAERR41:dibenzofuran differs significantly from that of BphAELB400:biphenyl, but is similar to that of BphAELB400:2-chlorobiphenyl (Fig. 5). This is caused principally by the replacement of Phe336 by Met combined with the conformational freedom of Gly321. The new catalytic properties of BphAELB400 toward dibenzofuran (and in part those of BphAERR41) can thus be attributed to structural changes in the distal portion of the substrate binding pocket. As noted for BphAELB400, the Thr335Ala mutation relieves constraints on the Val320:Gln322 segment allowing displacement of the Gly321 carbonyl such that it moves away from the substrate (17). In this case, the removal of Gly321 from dibenzofuran reduces the influence it exerts on the substrate’s distal ring. This is significant because dibenzofuran is obligatory co-planar: an altered placement of the distal ring would influence the orientation of the proximal ring inside the catalytic pocket.

**Structural analysis of the influence of residues 338 and 409 on enzyme stability**

Based on the crystal structure of BphAERR41, as well as the structures of BphAELB400 and BphAELB400 (17), Gln338 and Phe409 are too distant from each other to interact. In order to understand the effect of these two mutations we need to examine closely the overall structure of the α and β subunits and the contacts at αα, ββ, and αβ interfaces.

The overall crystal structure of the β subunit of BphAERR41 is very similar to that of other biphenyl dioxygenases (16, 17) and naphthalene dioxygenase (2). It includes a long twisted six-stranded β sheet, with three helices on the inward side of the sheet and a loop made of residues 9-20 (Fig. 6A) on its outward side. Many polar interactions are uniformly distributed between the β-sheet residues of vicinal β subunits and between two of the helices and β-sheet residues of the vicinal subunit. In addition, helix α3 and strand β2 are in contact with the α subunit. This suggests the β subunit plays a key role in subunit assembly.

As reported for other dioxygenases (2,16), the α subunit comprises two domains, the Rieske and catalytic domains. Unlike the β subunits, the crystal structures show unevenly distributed contacts between vicinal α subunits; these occur principally at the junction between the Rieske domain of one α subunit and the catalytic domain of its vicinal subunit (Fig 6A).

The Rieske domain is dominated by antiparallel β strands from which two hairpin structures protrude to form two fingers that hold the [2Fe—2S] center. The catalytic domain contains the catalytic Fe³⁺, which lies against a eight-stranded antiparallel β sheet on one side and is surrounded on the other sides by helices and loops (Fig. 6B). Residues Arg101, His102, Arg103 and Gly104 of the Rieske domain form the tip of hairpin 1. This short segment (in blue on Fig. 6B and C) is embedded inside a matching trough of the vicinal α subunit; it faces helix α6 of the catalytic domain, comprised of residues Trp220 to Ser226, and it also contacts a short segment (P21) comprised of residues Pro408, Phe409 and Asn410 and located at the edge of the catalytic domain. Arg103 forms a polar contact with Glu225 of helix α6. Arg101 forms
polar contacts with Pro\textsuperscript{408} and Asn\textsuperscript{410}, and Arg\textsuperscript{404} forms a polar contact with Asn\textsuperscript{410} (Fig. 6C). Therefore, the crystal structures show residue Phe\textsuperscript{409} is located within a stretch of amino acids that appears to play an important role in subunit assembly and/or in maintaining the stability of the oligomeric structure. In BphA\textsubscript{ERR41}, Phe\textsuperscript{409} is approximately 4.6 Å from Phe\textsuperscript{222} of helix \(\alpha\) (Fig. 6C). Alignment of dimers AB, CD, EF, GH, IJ and KL of BphA\textsubscript{ERR41} and its dibenzofuran-bound form with dimer AB, CD, EF, GH, IJ and KL of substrate-free and bound forms of BphA\textsubscript{ELB400} or BphA\textsubscript{p4} shows Phe\textsuperscript{409} of BphA\textsubscript{ERR41} aligns very well with Leu\textsuperscript{409} of BphA\textsubscript{ELB400} or BphA\textsubscript{p4} (not shown). In the latter enzymes, however, Leu\textsuperscript{409} is at an average distance of 5.4 Å from Phe\textsuperscript{222}. This could explain why replacing Leu\textsuperscript{409} of BphA\textsubscript{p4} with a larger side chain in Phe\textsuperscript{409} suppresses the negative impact of the Asn\textsuperscript{338}Gln mutation on hexamer assembly. Through its interaction with Phe\textsuperscript{222}, Phe\textsuperscript{409} seems to help stabilize subunit assembly by reinforcing the role played by segment Pro\textsuperscript{408}-Asn\textsuperscript{410} in holding the subunits together.

Structural analysis of the influence of residues 338 and 409 on catalytic properties- Prior studies identified mutations within a subsequence called region III that influenced the oxygenase’s catalytic properties (12-14). This region includes a loop between strands \(\beta\)18 and \(\beta\)19 and a portion of strand \(\beta\)19. Residues 338 and 341 are both located on strand \(\beta\)19 (Fig. 6C). Superposition of the catalytic domains of BphA\textsubscript{ERR41} and BphA\textsubscript{ELB400} reveals minor variations in strand \(\beta\)19 that can be attributed to the longer side chain of Gln\textsuperscript{338} in BphA\textsubscript{ERR41} (not shown). Strand \(\beta\)19 faces helix \(\alpha\)12, which interacts with the tip of hairpin-2 of the vicinal Rieske domain and includes Fe ligand Asp\textsuperscript{388}. The tip of Rieske domain hairpin-2 includes Ser\textsuperscript{121}, Tyr\textsuperscript{122} and His\textsuperscript{123}. These residues make polar contacts with Thr\textsuperscript{237} and Thr\textsuperscript{238} located at the junction between helices \(\alpha\)7 and \(\alpha\)8 on which are located Fe ligands His\textsuperscript{233} and His\textsuperscript{339}. In addition, Ser\textsuperscript{121} and His\textsuperscript{123} make polar contacts with Gln\textsuperscript{226} and Asp\textsuperscript{230}, two residues believed to be involved in the reaction mechanism (36,38) and found in the catalytic cavity at the level of the proximal ring of the substrate (17) (Fig. 6B). Furthermore, Tyr\textsuperscript{122} has a polar contact with Trp\textsuperscript{392} and Ser\textsuperscript{121} with Asn\textsuperscript{391} of helix \(\alpha\)12. Gln\textsuperscript{338} forms two polar contacts with Arg\textsuperscript{340} and the latter forms two polar contacts with Glu\textsuperscript{385} of helix \(\alpha\)12. Arg\textsuperscript{340} is also close enough from Glu\textsuperscript{385} to form a salt bridge with this residue (Fig. 6C). Therefore, Arg\textsuperscript{340} and Gln\textsuperscript{338} are located such that their conformation can influence the distribution in space of helices \(\alpha\)12, \(\alpha\)8, \(\alpha\)7 and \(\alpha\)6, which are critical for catalytic activity and subunit assembly.

Analysis of the crystal structure did not suggest a clear-cut mechanism by which Gln\textsuperscript{338} and Arg\textsuperscript{340} exert these effects. However, the longer length of Gln\textsuperscript{338} side chain compared to Asn\textsuperscript{338} might disturb a key state not observed in the crystal or the internal dynamics of the protein. With respect to the latter possibility, it is clear from structural analysis that residues on helix \(\alpha\)12 move considerably during substrate binding showing this protein segment is rather adaptable (Fig. 7). This movement is likely required to suitably align the reactive atoms for progression along the chemical reaction.

In ROs, the proximity between the Rieske cluster and the iron in the active site of the adjacent \(\alpha\) subunit is consistent with a mechanism involving a transfer of electron across the interface between two subunits (2,16,17). This is corroborated by the fact that full activity requires that \(\alpha\) and \(\beta\) subunits associate into a \(\alpha_3\beta_3\) configuration (31). Such a mechanism must demand precise alignment of the amino acids involved in electron transfer between the Rieske cluster and the mononuclear iron of each adjacent \(\alpha\) subunits highlighting the importance of the protein atoms involved in the \(\alpha\beta\) subunit interface.

Therefore, the crystal structure analysis is consistent with an induced-fit response required to reorganize the active site and facilitate the interplay between protein atoms critical for the reaction. The Asn\textsuperscript{338}Gln substitution might disturb the conformation of helices \(\alpha\)12 and \(\alpha\)6 resulting in subunit instability or misassembly. Conversely, the double Gln\textsuperscript{338}Phe\textsuperscript{409} mutation may affect the conformational fluctuations of these helices in such a way that it enhances the roles of protein residues such as Asn\textsuperscript{388}, Gln\textsuperscript{226} and Asp\textsuperscript{230} that are
located on the helices and presumed to be involved in the chemical steps of the reaction (2,38).

DISCUSSION

In this study we examined the crystal structural of BphAE<sub>RR41</sub>, an evolved RO that oxidizes dibenzofuran more efficiently than its BphAE<sub>LB400</sub> and BphAE<sub>p4</sub> parents. In spite of the limitations of crystal structure analyses, the study revealed two pathways through which ROs evolve to expand their substrate range.

Traditionally, enzyme engineering to alter the substrate range involves mutations at residues lining the catalytic pocket. This approach has been applied successfully in many circumstances (39-45). Reducing the size of a side chain or altering charge distributions can generate enzyme with new catalytic properties.

However, other studies have shown that several residues not in direct contact with the substrate can significantly change BPDO's catalytic properties toward biphenyl analogs (21,22,44). In this work we confirm the importance of the Thr<sup>335</sup>Ala mutation. In altering the plasticity of the catalytic cavity, this mutation allows the carbonyl of residue Gly<sup>321</sup> to move away from the substrate. In a previous work, we showed this movement was required to increase the space available to bind the bulky 2,6-dichlorobiphenyl in a productive orientation (17). Because dibenzofuran is obligatory co-planar, any misplacement of the distal ring would influence the orientation of the proximal ring inside the catalytic pocket. Therefore, consistent with an induced-fit mechanism, in BphAE<sub>p4</sub> and BphAE<sub>RR41</sub>, the displacement of Gly<sup>321</sup> appears to be required to reduce the influence it exerts through atomic interactions on the substrate’s distal ring.

In this work, we highlighted a second and more subtle route to changes in substrate range, which implies that in ROs, either one or both of the induced-fit or protein dynamic processes are involved to place the protein atoms involved in the reaction into proper relationships that facilitate catalysis. The reaction catalyzed by ROs is complex; it not only involves substrate binding and release of product, but also one dioxygen molecule is required in the reaction and electrons must be transferred from the ferredoxin component to the Rieske cluster of one α subunit and then to the catalytic iron of the vicinal α subunit. Furthermore, a recent report showed residues at the interface between the Rieske domain and the catalytic domain move during formation of the complex between the carbazole 1,9a-dioxygenase’s oxygenase and ferredoxin components (24). This implies reaction-critical atoms from the Rieske domain must align properly with those of the vicinal catalytic domain and the reaction-critical atoms of the catalytic domain must align properly to work together during the catalytic process. Structural analysis shows residues located on secondary structures α6 and α12 are involved in subunit assembly and biochemical data suggest they are involved in the catalytic reaction (electron transfer and protonation) (2,38). The fact that these residues move during substrate binding is consistent with a substrate-induced retuning process required to suitably align the protein atoms involved in the chemical steps of the reaction. In such a context, by altering the interactions occurring between secondary structure elements surrounding the catalytic center, the Asn<sup>338</sup>Gln mutation generates a protein unable to stabilize the α<sub>6</sub>β<sub>3</sub> assembly previously shown to be required for activity (31). However, the double Asn<sup>338</sup>Gln and Leu<sup>409</sup>Phe substitution generates an α subunit that supports a stable hexamer and where the retuning process is improved compared to its BphAE<sub>LB400</sub> and BphAE<sub>p4</sub> parents, resulting in a more efficient and faster catalytic reaction. ROs can thus be engineered to enhance their catalytic properties toward new substrates by altering the process involved in fine-tuning the interplay between the reaction-critical atoms.

Many questions remain unanswered; crystal structure analysis did not determine a clear-cut mechanism by which the double Asn<sup>338</sup>Gln Leu<sup>409</sup>Phe substitution affects the enzyme structure and catalytic properties, and our data do not determine which of the enzymatic steps are accelerated during the reaction. Although the data do not provide any direct demonstration that the Asn<sup>338</sup>Gln and Leu<sup>409</sup>Phe substitutions either affect an induced-fit or protein dynamic mechanism
involved in the catalytic reaction, it is clear from crystal structure analysis that these residues occupy strategic positions whereby they can interact with reaction-critical protein atoms/groups and affect oligomeric assembly. Furthermore, residues of helix $\alpha_{12}$ and $\alpha_6$, and especially Asp$^{388}$ and Gln$^{226}$, which are postulated to play a key role in the catalytic reaction (36) moved significantly during substrate binding in all variants (BphAE$_{LB400}$, BphAE$_{pl}$ and BphAE$_{RB41}$).

Altogether, our analysis shows that evolving ROs to change their substrate specificity is a rather complex enterprise that does not involve exclusively mutations at key residues in direct contact with the substrate. It appears that some mutations affect key residues associated with necessary conformational changes that are more difficult to identify by a rational approach, but that are required to allow productive or improved interplay of reaction-critical atoms both inside and outside of the substrate binding pocket.

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FOOTNOTES

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Abbreviations: BPDO, biphenyl dioxygenase; GC-MS gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; NDO, naphthalene dioxygenase; PCB, polychlorinated biphenyl; RO, Rieske-type oxygenase; SDS-PAGE sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

FIGURES LEGENDS

Fig. 1. Biphenyl dioxygenase reaction. The inset shows the structure of dibenzofuran and possible positions of oxygenation, A (angular), L (lateral).

Fig. 2. GC-MS spectra showing the relative amounts of the dihydrodihydroxy metabolites produced from dibenzofuran by BphAE_{LB400} and its variants. 1.2 nmol of each purified enzyme preparation was incubated for 2 min under the conditions described in the Experimental Procedures. The metabolites were extracted and derivatized with butylboronate before analysis by GC-MS.

Fig. 3. SDS-PAGE of lyzates of IPTG-induced E. coli cells expressing the indicated variants derived from BphAE_{p4} (T335A/F336M). A. Gel of the indicated protein extracts prepared under denaturing conditions. B. Gel of the indicated protein extracts prepared under non-denaturing conditions. In addition to the indicated substitutions, all mutants in lane 4-8 carry the double T336A/F336M mutation. The left lane is the molecular weight marker, the bands corresponding to the \( \alpha \) and \( \beta \) subunits are marked by an arrow.

Fig. 4. The \( 2F_{\text{obs}}-F_{\text{calc}} \) electron density contoured at 1.0 \( \sigma \) level in the vicinity of the active site of chain AB from BphAE_{RR41}. A. Substrate-free. B. Dibenzofuran-bound form.

Fig. 5. Superposition of a portion of the catalytic cavity showing the residues lining the distal ring pocket. Superposition of dimers AB of A. BphAE_{RR41}:dibenzofuran (yellow) and BphAE_{LB400}:biphenyl (red) and B. BphAE_{RR41}:dibenzofuran (yellow) and BphAE_{p4}:2,6-dichlorobiphenyl (green).

Fig. 6. A. Backbone ribbon drawing of the vicinal \( \alpha/\beta \) dimers AB (green and salmon) and CD (red and purple) of BphAE_{RR41}:dibenzofuran highlighting the numerous contacts between two vicinal \( \beta \) subunits. B. Ribbon drawing of the catalytic domain of monomer C (red) and of the Rieske domain of monomer A (green) of BphAE_{RR41}:dibenzofuran highlighting the interface between the two monomers. C. Close-up view of the same interface shown in (B). Hairpins 1 and 2 from the Rieske domains of monomer A (in blue and grey) protrude into a matching trough of the vicinal catalytic domain of monomer C. The residues from monomer C that contact the hairpins from the vicinal subunit are coloured in blue or grey to match the residues they contact.
Fig. 7. Superposition of residues of the catalytic domain that move after substrate binding. A. Residues of BphAE<sub>RR41</sub> (red) and BphAE<sub>RR41</sub>:dibenzofuran (green). B. Residues of BphAE<sub>LB400</sub> (brown) and BphAE<sub>LB400</sub>:biphenyl (yellow). C. Residues of BphAE<sub>p4</sub> (grey) and BphAE<sub>p4</sub>:2,6-dichlorobiphenyl (purple).
Table 1  
Sequence pattern of BphAE_{LB400} variants

| Protein designation | Residue position$^1$ |
|---------------------|----------------------|
|                     | 335  | 336  | 338  | 341  | 409  |
| BphAE_{LB400}       | T    | F    | N    | I    | L    |
|                     | A    | F    | N    | I    | L    |
|                     | T    | M    | N    | I    | L    |
| BphAE_{pd}          | A    | M    | N    | I    | L    |
|                     | A    | M    | Q    | I    | L    |
|                     | A    | M    | N    | V    | L    |
|                     | A    | M    | N    | I    | F    |
|                     | A    | M    | Q    | V    | L    |
|                     | A    | M    | N    | V    | F    |
|                     | A    | M    | Q    | I    | F    |
| BphAE_{RR41}        | A    | M    | Q    | V    | F    |

$^1$All other residues for these variants are identical to those of BphAE_{LB400}
Table 2
Steady-state kinetic parameters\(^1\) of BPDO variants

| Substrate | BphAE\(_{LB400}\) | BpbAE\(_{p4}\) | BphAE\(_{R41}\) |
|-----------|-----------------|----------------|----------------|
|           | WT T335A F336M | T335A/F336M I341V \(^2\) | N338Q/L409F \(^2\) |
| Biphenyl   |                 |                |                 |
| \(K_m\) (\(\mu\)M) | 22 (0.0) 30 (4.0) 17 (5.0) | 33 (1.4) 32.5 (2.0) 21.5 (0.7) | 32.0 (0.0) 34.5 (6.3) |
| \(k_{cat}\) (s\(^{-1}\)) | 0.9 (0.1) 1.1 (0.0) 0.4 (0.1) | 1.0 (0.1) 0.7 (0.1) 0.8 (0.1) | 1.5 (0.1) 1.3 (0.3) |
| \(k_{cat}/K_m\) (10\(^3\) M\(^{-1}\) s\(^{-1}\)) | 41 (6.0) 36 (5.0) 23 (0.1) | 31 (4) 21 (0.5) 37 (3.0) | 46 (4.0) 38 (0.0) |
| Dibenzo furan |               |                |                 |
| \(K_m\) (\(\mu\)M) | 19.5 (0.7) 19 (0.6) 19 (1) | 20 (1.4) 25 (9.9) 21.5 (2.1) | 23 (7.8) 22.0 (0.0) |
| \(k_{cat}\) (s\(^{-1}\)) | 0.12 (0.0) 0.22 (0.0) 0.12 (0.05) | 0.26 (0.01) 0.17 (0.07) 0.22 (0.02) | 0.5 (0.05) 0.38 (0.08) |
| \(k_{cat}/K_m\) (10\(^3\) M\(^{-1}\) s\(^{-1}\)) | 6 (0.0) 12 (2.0) 6 (0.07) | 13 (2.0) 7 (0.1) 10 (0.1) | 22 (5) 17 (4.0) |

\(^1\)The steady-states kinetics were determined from the oxygen consumption rates as described in the Experimental Procedure section.

\(^2\)These mutants carry also the double T335A/F336M mutation.
Table 3
Crystallographic data and refinement results for BphAE_{RR41} structure

|                          | BphAE_{RR41} | BphAE_{RR41}: dibenzofuran |
|--------------------------|--------------|----------------------------|
| Crystallographic data    |              |                            |
| Space group              | $P2_1$       | $P2_1$                     |
| Wavelength               | 0.9          | 0.9                        |
| Resolution               | 100-2.2      | 100-2.2                    |
| Cell dimensions          |              |                            |
| $a$ (Å)                  | 86.9         | 86.9                       |
| $b$ (Å)                  | 277.8        | 278.1                      |
| $c$ (Å)                  | 92.9         | 92.9                       |
| $\alpha$ (°)             | 90.0         | 90.0                       |
| $\beta$ (°)              | 117.6        | 117.6                      |
| $\gamma$ (°)             | 90.0         | 90.0                       |
| Unique reflections       | 208106       | 210175                     |
| Completeness (%) (Last shell) | 99.0 (94.0) | 92.9 (80.3)                |
| $R_{sym}$ (%) (Last Shell) | 7.0(2)     | 8.0 (55.0)                  |
| $I/\sigma$ (Last shell)  | 17.4 (2.2)  | 16.0 (2.0)                  |
| Multiplicity (Last shell) | 3.7 (3.0)  | 4.4 (2.5)                   |
| Refined model            |              |                            |
| No. of residues          | 3720         | 3720                       |
| Water molecules          | 1217         | 1287                       |
| Resolution range (Å)     | 100-2.2      | 100-2.2                    |
| $R_{fact}$ (%)           | 17.6         | 19.7                       |
| $R_{free}$ (%)           | 22.2         | 22.9                       |
| Average B-factors (Å$^2$)|              |                            |
| Protein chains           | AB 42.4, 43.2 | AB 42.3, 42.5    |
|                          | CD 42.6, 43.3 | CD 42.2, 42.7    |
|                          | EF 44.7, 44.0 | EF 43.4, 43.2    |
|                          | GH 47.1, 45.5 | GH 42.2, 42.9    |
|                          | IJ 47.7, 46.9 | IJ 42.9, 42.4    |
|                          | KL 48.4, 45.9 | KL 42.9, 41.6    |
| Waters                   | 49.4         | 42.6                       |
| All atoms                | 30803        | 30992                      |
| Bond lengths (Å)         | 0.01         | 0.01                       |
| Bond angles (°)          | 1.33         | 0.91                       |
| Ramachandran plot (%)    | Preferred    | 89.3                       |
|                          | Allowed      | 10.6                       |
|                          | Outliers     | 0.1                        |

\[ R_{sym} = \frac{\sum_{hk\ell} \sum_{i=1}^n |I_{hk\ell,i} - \bar{I}_{hk\ell}|}{\sum_{hk\ell} \sum_{i=1}^n I_{hk\ell,i}} \]
Figure 1

NAD$^+$ → NADH+H$^+$

(ox) BphG → (red) BphF

(ox) BphF → (red) BphAE

O$_2$

cis-(2R,3S)-dihydroxy-1-phenylcyclohexa-4,6-diene

p-dibenzofuran
Figure 3

A

B

BphAE<sub>cry</sub>  BphAE<sub>mut</sub>  N338Q/I341V  I341V/L409F  I341V  N338Q  N338Q/L409F

α

β

BphAE<sub>cry</sub>  BphAE<sub>mut</sub>  N338Q/I341V  I341V/L409F  I341V  N338Q  N338Q/L409F

α

β
Retuning rieske-type oxygenases to expand substrate range
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