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Metabolism of chlorobiphenyls by a variant biphenyl dioxygenase exhibiting enhanced activity toward dibenzofuran

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ABSTRACT

The biphenyl dioxygenase of *Burkholderia xenovorans* LB400 ($BphAE_{LB400}$) catalyzes the dihydroxylation of biphenyl and of several polychlorinated biphenyls (PCBs) but it poorly oxidizes dibenzofuran. In this work we showed that $BphAE_{RR41}$, a variant which was previously found to metabolize dibenzofuran more efficiently than its parent $BphAE_{LB400}$, metabolized a broader range of PCBs than $BphAE_{LB400}$. Hence, $BphAE_{RR41}$ was able to metabolize 2,6,2′,6′-, 3,4,3′,5′- and 2,4,3′,4′-tetrachlorobiphenyl that $BphAE_{LB400}$ is unable to metabolize. $BphAE_{RR41}$ was obtained by changing Thr335Phe336Asn338Ile341Leu409 of $BphAE_{LB400}$ to Ala335Met336Gln338Val341Phe409. Site-directed mutagenesis was used to create combinations of each substitution, in order to assess their individual contributions. Data show that the same Asn338Glu/Leu409Phe substitution that enhanced the ability to metabolize dibenzofuran resulted in a broadening of the PCB substrates range of the enzyme. The role of these substitutions on regiospecificities toward selected PCBs is also discussed.

1. Introduction

Aryl hydroxylating Rieske-type dioxygenases (ROs) catalyze a *cis* dioxygenation of aryl compounds (Fig. 1) to generate a *cis*-dihydrodiol metabolite. ROs are promising biocatalysts that metabolize many substituted benzene or diphenyl rings as well as bicyclic- or tricyclic-fused heterocyclic aromatics such as quinoline, dibenzofuran, and flavonoids [1–7].

The biphenyl dioxygenase (BPDO) which catalyzes the first reaction of the bacterial biphenyl catabolic pathway is a RO that metabolizes several polychlorinated biphenyl (PCB) congeners. BPDO comprises three components [8–10]. The catalytic component (BphAE) is a hetero hexamer made up of three α (BphA, a RO protein) and three β subunits (BphE). The ferredoxin (BphF) and the ferredoxin reductase (BphG) are involved in electron transfer from NADH to BphAE. The encoding genes for both *Burkholderia xenovorans* BPDO are *bphA* ($BphAE_{LB400}$ α subunit), *bphE* ($BphAE_{LB400}$ β subunit), *bphF* (BphF) and *bphG* (BphG) [11,12]. BphAE interacts with the substrate to catalyze a 2,3-dihydroxylation. The α subunit is the one involved in the catalytic activity and it comprises two domains [10]. The Rieske domain containing a 2Fe-2S Rieske cluster receives the electrons from BphF and transfers them to the catalytic mononuclear iron center of the catalytic domain [13]. It is noteworthy that the Rieske cluster and the catalytic iron are too far from each other inside the α subunit to interact together, therefore the electron transfer during the catalytic reaction proceeds from the ferredoxin to the Rieske cluster of one α subunit and then to the catalytic iron of the vicinal α subunit [13].

Many investigations have provided evidences the substrate range and regiospecificity are principally determined by residues located on the C-terminal portion of BphAE, some of which are in contact and other are removed from it [13–18]. In recent years, using semi-rational directed evolution approaches targeting the residues of the C-terminal portion of $BphAE_{LB400}$, $BphAE_{p4}$ and $BphAE_{RR41}$ variants were obtained that were used to get more insight into the BPDO reaction [13,19–21]. $BphAE_{p4}$ was obtained by the double substitution of $BphAE_{LB400}$ Thr335Phe336 to Ala335Met336. Compared to $BphAE_{LB400}$, $BphAE_{p4}$ exhibits an expanded PCB substrate range and an enhanced ability to metabolize the co-planar dibenzofuran [19]. $BphAE_{RR41}$ was obtained by changing Asn338Ile341Leu409 of $BphAE_{p4}$ to Gln338Val341Phe409 [21]. $BphAE_{RR41}$ exhibits an enhanced ability to metabolize dibenzofuran compared to $BphAE_{p4}$ [21]. On the bases of biochemical and structural analyses of the parental and variant enzymes, we

Abbreviations: BPDO, biphenyl dioxygenase; GC–MS, gas chromatography–mass spectrometry; CB, chlorobiphenyl; PCB, polychlorinated biphenyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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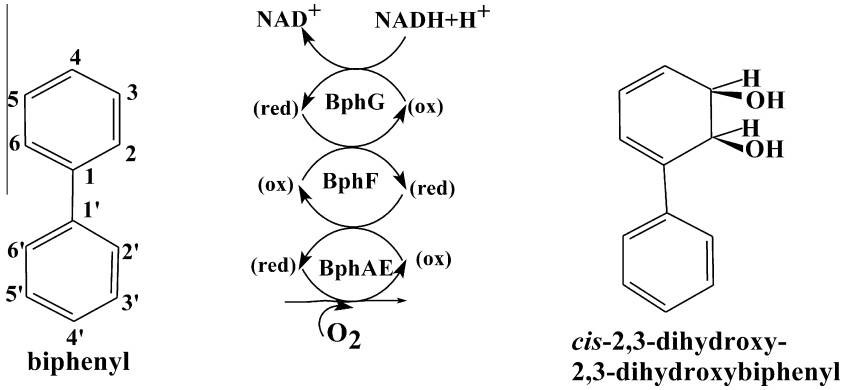

Fig. 1. The biphenyl dioxygenase reaction.

showed that Thr335 of $BphAE_{LB400}$ imposes constraints through polar contacts to the segment Val320Gly321Gln322 lining the catalytic pocket [20]. Changing Thr335 to Ala relieves intramolecular constraints allowing for significant movement of these residues during substrate binding, thereby increasing the space available to accommodate bulkier substrates such as 2,6-dichlorobiphenyl or more stringent ones such as dibenzofuran. The superior ability of $BphAE_{RR41}$ to metabolize dibenzofuran over $BphAE_{p4}$ was attributed to the double substitution Asn338Gln and Leu408Phe which are both essential. Both residues are far from the substrate, but together, they influence the subunit assembly and a substrate-induced retuning of reaction-critical atoms to suitably align them during the catalytic reaction [13].

The mutations that occurred in $BphAE_{p4}$ to change its substrate range have also altered their regiospecificity toward *ortho*-substituted PCBs [21,22]. Structural and biochemical analysis identified Phe336 as a key determinant of regiospecificity toward chlorobiphenyls [13]. This residue interact directly with the substrate and thus influences its orientation inside the catalytic pocket.

Although $BphAE_{RR41}$ exhibited enhanced ability to metabolize dibenzofuran, its catalytic properties toward chlorobiphenyl has never been evaluated. The purpose of this work was to compare the catalytic properties of $BphAE_{RR41}$ and of $BphAE_{p4}$ toward chlorobiphenyls. Data show the mutations that have increased the turnover rate of $BphAE_{RR41}$ toward dibenzofuran has also enhanced the ability of this enzyme to metabolize several PCB congeners that $BphAE_{LB400}$ and $BphAE_{p4}$ metabolize poorly.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli DH11S [23] was used in this study. *E. coli* DH11S clones expressing from pQE31, $BphAE_{LB400}$ or its mutants listed in Table 1 were described previously [13]. These recombinant cells were transformed with pDB31[*bphFG*-LB400] cloned from *B. xenovorans* LB400 as described previously [24].

2.2. Whole cell assays to determine the ability of variant BphAEs to metabolize PCBs

Isopropyl-β-D-thiogalacto-pyranoside (IPTG)-induced *E. coli* DH11S pDB31[*bphFG*-LB400] cells harbouring appropriate variants of pQE31[*bphAE*] were tested for their ability to degrade a synthetic mix of 18 PCBs using a protocol published previously [24]. The synthetic mix comprised 1 μM of each of the following congeners: 2,6-dichlorobiphenyl (CB), 2,4,3′-CB, 2,4,4′-CB, 2,3,4′-CB, 2,6,2′,6′-CB, 2,5,2′,5′-CB, 2,4,2′,4′-CB, 2,3,2′,3′-CB, 2,4,3′,4′-CB, 3,4,3′,4′-CB, 3,4,3′,5′-CB, 2,3,4,2′,5′-CB, 2,4,5,3′,4′-CB, 2,4,5,2′,4′,5′-CB, 2,3,4,5,2′5′-CB, and 5 μM of 3,3′-CB and 4,4′-CB. The internal standard 2,3,4,5,6,2′,3′,5′,6′-CB, presumed to be not degraded, was present at a concentration of 1 μM. Each experiment included control cultures of *E. coli* [pDB31*bphFG*-LB400] [pQE31] that were run under conditions identical to the experimental cultures. All of the values reported in the present study are averages from triplicate experiments.

PCB metabolites were analyzed from suspensions of IPTG-induced cells of *E. coli* DH11S pDB31[*bphFG*-LB400] pQE31[*bphAE*] according to a protocol described previously [19]. In this case, log phase cells grown in LB broth were induced for 2 h with 0.5 mM IPTG and then washed and suspended to an optical density at 600 nm of 2.0 in M9 medium [25] containing 0.5 mM IPTG. The cell suspension was distributed by portions of 2 ml. Each tube received 10 μl of a 50 mM acetone solution of the appropriate chlorobiphenyl substrate. They were incubated for 18 h at 37 °C with shaking. Cell suspensions were extracted at neutral pH with ethyl acetate. The metabolites were identified by gas chromatography–mass spectrometry (GC–MS) analyses of their butylboronate or trimethylsilyl derivatives [19]. The sums of the area under the peak of metabolites obtained in these experiments were used to calculate the relative activity of each variant enzyme toward the tested chlorobiphenyls. The catalytic activity of each variant was expressed relative to the activity of IPTG-induced *E. coli* DH11S pDB31[*bphFG*-LB400] pQE31[*bphAE*-LB400] cells tested under identical conditions. The levels of expression of each variant enzyme by the recombinant *E. coli* clones were assessed from SDS–PAGE [26] of crude cell extracts stained with Coomassie Brilliant Blue and they were found to be similar for all strains.

Table 1
Sequence pattern of $BphAE_{LB400}$ variants used in this study.

Variant designation	Residue position[a]				
	335	336	338	341	409
$BphAE_{LB400}$	Thr	Phe	Asn	Ile	Leu
	Ala	Phe	Asn	Ile	Leu
	Thr	Met	Asn	Ile	Leu
$BphAE_{p4}$	Ala	Met	Asn	Ile	Leu
	Ala	Met	Gln	Ile	Leu
	Ala	Met	Asn	Val	Leu
	Ala	Met	Asn	Ile	Phe
	Ala	Met	Gln	Val	Leu
	Ala	Met	Asn	Val	Phe
	Ala	Met	Gln	Ile	Phe
$BphAE_{RR41}$	Ala	Met	Gln	Val	Phe

[a] All other residues for these variants are identical to those of $BphAE_{LB400}$.