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Biphenyl hydroxylation enhanced by an engineered *o*-xylene dioxygenase from *Rhodococcus* sp. strain DK17

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Abstract

Hydroxylation of the non-growth substrate biphenyl by recombinant *o*-xylene dioxygenases from *Rhodococcus* sp. strain DK17 was studied through bioconversion experiments. The metabolites from the biphenyl hydroxylation by each enzyme were identified and quantified by gas chromatography-mass spectrometry. The L266F mutant enzyme produced much more 2-hydroxybiphenyl (2.43 vs. 0.1 μg/L) and 3-hydroxybiphenyl (1.97 vs. 0.03 μg/L) than the wild-type. Site-directed mutagenesis combined with structural and functional analyses indicated that hydrophobic interactions and shielding effects against water are important factors in the hydroxylation of biphenyl by the *o*-xylene dioxygenase. The residue at position 266 plays a key role in coordinating the reaction.

Keywords: *Rhodococcus*; Hydrophobic interactions; Aromatic hydroxylation; Rieske dioxygenase

1. Introduction

Rhodococcus sp. strain DK17 can metabolize various benzene derivatives including *o*-xylene, toluene, ethylbenzene, and indane, by degrading them through a common pathway initiated by *o*-xylene dioxygenase, a ring-oxidizing dioxygenase (Kim et al., 2010, 2002). The DK17 *o*-xylene dioxygenase enzyme possesses the unique ability to perform distinct regioselective hydroxylations that depend on the position and the size of the substituent groups on the aromatic ring (Kim et al., 2004, 2007). For example, the enzyme catalyzes dioxygenation on the aromatic ring of *o*-xylene to produce only the *o*-xylene *cis*-3,4-dihydrodiol form of dihydrodiol or on ethylbenzene to produce the two dihydrodiols, ethylbenzene *cis*-2,3- and *cis*-3,4-dihydrodiol. The same enzyme has also been reported to oxidize *m*-xylene into 3-methylbenzylalcohol and 2,4-dimethylphenol at a ratio of 9:1 (Kim et al., 2010).

The DK17 *o*-xylene dioxygenase, which belongs to the Rieske oxygenase class of enzymes (Ferraro et al., 2005; Kwon et al., 2008), consists of a reductase (AkbA4) component, a ferredoxin (AkbA3) component, and a Rieske oxygenase component that contains large and small subunits (AkbA1A2) (Kim et al., 2004). A molecular modeling study based on the crystal structure of the biphenyl dioxygenase large subunit from *Rhodococcus* sp. strain RHA1, the most homologous counterpart of the dioxygenase from strain DK17 among related enzymes with available crystal structures (Furusawa et al., 2004), predicted that A218, D262, L266, and V297 of AkbA1 affect the positioning of the substrate. A subsequent site-directed mutagenesis study demonstrated that A218 in the α7 helix and D262 in the α9 helix play important roles in positioning *m*-xylene (Kim et al., 2010). In contrast, mutating the other two residues did not result in readily detectable phenotypic changes in spite of those residues being modeled as being in proximity to either of the methyl groups of *m*-xylene

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(Fig. 1A). However, because the substrate binding pocket of AkbA1 is predicted to be more than large enough for monocyclics including xylene isomers, it is possible that the two amino acid residues have potential roles in the hydroxylation of bulkier substrates including biphenyl (Fig. 1B). This possibility led us to investigate if whether these residues could be implicated in the biphenyl hydroxylating activity of the DK17 *o*-xylene dioxygenase. Here, we report studies and functional modeling data on biphenyl hydroxylation by wild-type and engineered *o*-xylene dioxygenases.

2. Materials and methods

2.1. Preparation of expression clones for wild-type and mutant o-xylene dioxygenases

The wild-type and mutant enzymes (A218L, D262L, L266F, and V297L) were previously described (Kim et al., 2004, 2010). The expression host, *Escherichia coli* BL21(DE3), was first cotransformed with the pKJE7 chaperone plasmid (Takara Korea, Korea), which allows higher expression and stable maintenance of *o*-xylene dioxygenase (Kim et al., 2007). A negative control strain was also constructed by transforming *E. coli* BL21(DE3) with the same pEXP5-CT/TOPO vector used to construct the recombinant plasmids. The L266Y mutant was constructed via overlap extension PCR as principally described by Sakamoto et al. (2001). The first-round PCR reaction used: (1) the AkbA1-forward primer (5′-ATGGAGTGGAGCATGTTG-3′) and L266Y-MID reverse (5′-GGCGACCCGTACAAGCCGATAT-3′) to amplify the 5′ portion of the *akbA1A2A3* genes, and (2) the AkbA3-reverse primer (5′-TCATTGAGACTCGGCGCC-3′) and L266Y-MID forward primer (5′-ATATCGGCTTGTACGGGTCGCC-3′) to amplify the 3′ portion of the genes. We combined 1 μl from each PCR reaction for a second PCR reaction using only the AkbA1-forward and AkbA3-reverse primers. The final PCR products were cloned using the pCRT7/CT-TOPO TA expression kit (Invitrogen, USA). The thermal cycling program had a 10-min hot start (95 °C), 30 cycles of 30 s of denaturation (95 °C), 30 s of annealing (55 °C), 1 min of extension (72 °C),) and a final 10 min of extension (72 °C). The introduced mutations were confirmed by DNA sequencing.

2.2. Biotransformation experiments

One colony of each clone was inoculated into 20 ml LB medium with 100 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol, and then incubated overnight at 37 °C. Four milliliters of the overnight culture was transferred to 200 ml of the same medium and incubated under the same conditions. Once the cells reached an OD_{600} of approximately 0.6, protein expression was induced with 1.0 mM IPTG and 0.002% arabinose and the cells were then further incubated for 2 h at 37 °C. Cells were harvested by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.4), and resuspended in 40 ml of the same buffer supplemented with 20 mM glucose plus carbenicillin and chloramphenicol. Biphenyl was added at a final concentration of 100 mg/L, while *o*-xylene was provided in the vapor phase. The bioconversion was carried out at 30 °C for 12 h.

2.3. Structural identification of biphenyl metabolites

E. coli cells were pelleted by centrifugation at 10,000 × *g* for 30 min. A total of 50 μg of 3,4-dimethylphenol was added to the supernatant as an internal standard for the next extraction step. The sample was extracted twice with an equal volume of ethyl acetate and dried by a rotary evaporator. Two

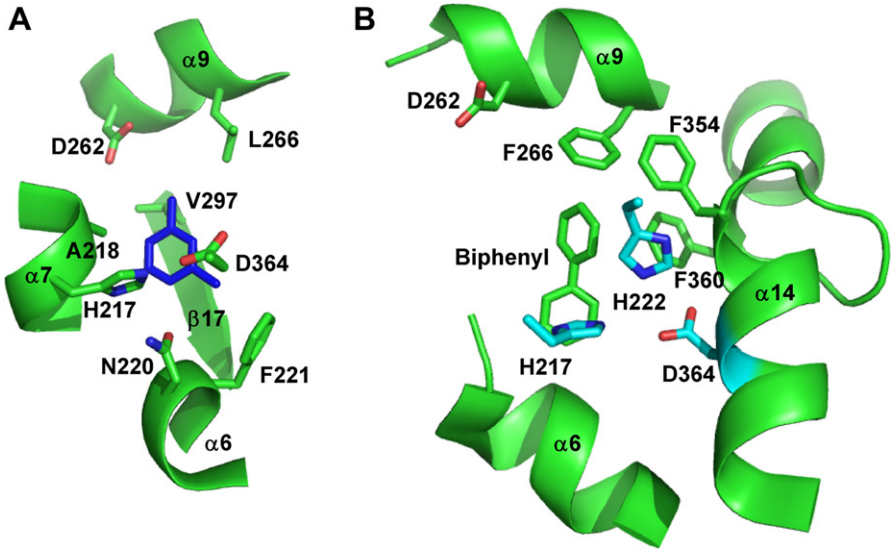

Fig. 1. Molecular modeling of substrate binding in the DK17 AkbA1. A. *m*-Xylene interaction [adapted and modified from Kim et al. (2010)]. B. Interactions between the biphenyl substrate and hydrophobic residues at the active site of the L266F mutant enzyme. Green sticks represent the substrate and the side chains from residues F266, F354, and F360. The residues H217, H222, D364 are iron ligands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

types of analyses were performed; the first one, an analysis for the dihydrodiol by extracting extraction and derivatizing on; and the second one, an analysis for the hydroxybiphenyls by dehydrating and extracting. For the first analysis, the dried metabolites were dissolved in methanol and treated with methane boronic acid in pyridine at 80 °C for 20 min to produce the methane boronate derivative as described previously (Kim et al., 2002). For the second analysis, the supernatant was acidified to pH 3.0 with an HCl and heat-treated at 70 °C for 30 min, which dehydrated the resultant resulting *cis*-dihydrodiol into two types of phenolic compounds (Omori et al., 1991).

Both the derivatized and non-derivatized original metabolites were analyzed by GC–MS with a Perkin Elmer Clarus 500 MS (electron impact ionization, 70 eV) connected to a Clarus 500 GC with an Elite-5 capillary column (0.25 mm × 30 m, 0.25-μm film thickness). The GC conditions were: 1.5 ml He/min; conventional split/splitless injector (CAP, injector temperature, 250 °C); and oven temperature at 100 °C for 1 min, increased to 250 °C at a rate of 10 °C/min, and then held at 250 °C for 10 min. The GC interface inlet line was 200 °C and the ionization temperature was 180 °C.

2.4. Molecular modeling

The molecular structure of the large subunit of the Rieske oxygenase component of the DK17 *o*-xylene dioxygenase was predicted using the Swiss-Model Server (Schwede et al., 2003) based on the crystal structure of biphenyl oxygenase from *Rhodococcus* sp. strain RHA1 (PDB ID 1ULI).

3. Results and discussion

The *o*-xylene dioxygenase enzyme from DK17 has been previously reported to hydroxylate biphenyl exclusively to *cis*-2,3-biphenyl dihydrodiol. However, DK17 cannot use biphenyl as its sole carbon and energy source (Kim et al., 2007). We investigated the function of the four AkbA1 amino acids of interest, A218, D262, L266, and V297, in the hydroxylation of biphenyl by expressing the previously constructed mutant enzymes (A218L, D262L, L266F, and V297L) in *E. coli* in the presence of biphenyl. Then, the metabolites were examined by GC/MS after being derivatized with methane boronic acid. SDS-PAGE analyses showed that *o*-xylene dioxygenase protein expression levels in each mutant and control were essentially equal (data not shown). The metabolites from the mutant enzymes had mass spectral patterns identical to each other and to the wild-type enzyme. The methane boronate derivatives of *cis*-2,3-biphenyl dihydrodiol with molecular ions at *m/z* 212 $[M]^+$ and conclusive ions at *m/z* 197 $[M-CH_3]^+$ had the expected fragmentation patterns. Our comparison of the peak areas of the metabolites revealed an interesting result: the L266F enzyme hydroxylated biphenyl at a much higher rate than the other mutant enzymes or the wild-type. We next quantified the *cis*-2,3-biphenyl dihydrodiol formed by each mutant enzyme and by the wild-type enzyme.

We replaced the derivatization step by a dehydration step. Measuring the dehydration products (either 2- or 3-hydroxybiphenyl) rather than directly measuring *cis*-2,3-biphenyl dihydrodiol is more reliable, because commercially available standards can be used. Besides the biphenyl parent compound peak at 5.42 min, which comprised over 90% of the total peak area depending on the activity of the respective clones, the GC–MS profiles of all the samples showed three major peaks: at 3.52 min from the internal standard, at 7.05 min from biphenyl metabolite I, and at 9.31 min from biphenyl metabolite II. The two biphenyl metabolites had the same molecular ions at *m/z* 170 from the base peak ion, and had significant ions at *m/z* 141 and 115 due to slightly different mass fragmentation patterns. Our subsequent GC–MS library search and comparison with authentic compounds successfully identified biphenyl metabolite I as 2-hydroxybiphenyl and biphenyl metabolite II as 3-hydroxybiphenyl.

The target peak area ratios from each sample were normalized to their respective internal standard and then converted to absolute amounts by reference to values obtained with the authentic 2- or 3-hydroxybiphenyl. As summarized in Fig. 2A, the L266F mutant enzyme produced much more 2-hydroxybiphenyl (2.43 μg/L vs. 0.1 μg/L) and 3-hydroxybiphenyl (1.97 vs. 0.03 μg/L) than the wild-type. The A218L mutant slightly increased metabolite production, while the hydroxylation activities of D262L decreased by approximately 50% and those of V297L by 90%.

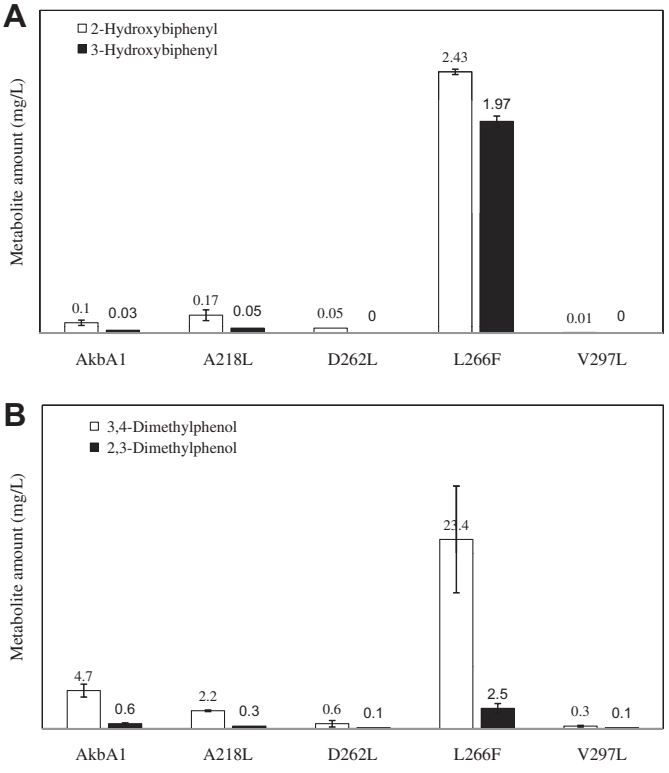

Fig. 2. Comparison of the amounts of biphenyl (A) and *o*-xylene (B) metabolites produced by the wild-type and mutant enzymes. Numbers above each bar represent the amount of each metabolite, which are averaged from at least three independent experiments. Error bars indicate standard deviations.

A recent functional modeling study suggested that the DK17 *o*-xylene dioxygenase holds *m*-xylene in a kinked region between the α6 and α7 helices of the active site, while the substrate is covered by the α9 helix (Fig. 1A) (Kim et al., 2010). The study found that the L266F mutation, which substitutes the bulkier hydrophobic side chain, did not affect the positioning of *m*-xylene, although the side chain was modeled as being close to either methyl group of *m*-xylene. In contrast, the positioning of biphenyl would be changed by the phenyl ring of the mutant enzyme L266F. This is modeled in Fig. 1B with the phenyl ring being positioned almost perpendicular to the aromatic ring of biphenyl that is distal to the α6 helix. This configuration apparently enhances the hydrophobic interactions between the two ring structures. Thus, combined with the fact that the L266F mutant enzyme also hydroxylates *o*-xylene at much higher rates than the wild-type enzyme (Fig. 2B), it is likely that the change at position 266 from leucine to phenylalanine increases the binding stability between L266F and biphenyl via hydrophobic interactions.

We corroborated this hypothesis by generating and characterizing an additional mutant enzyme that replaced L266 with tyrosine. The L266Y enzyme produced 72% of the biphenyl metabolites produced by the wild-type enzyme and 2% of those produced by the L266F enzyme. The introduction of a hydroxyl group at position 266 hinders the hydrophobic interactions needed for optimal substrate binding and dramatically changes the activity of the L266F mutant, as suggested by the results with the L266Y enzyme and by the fact that the only structural difference between tyrosine and phenylalanine is the hydroxyl group on the phenyl ring of tyrosine.

Modeling suggests that the four hydrophobic phenyl ring structures at the active site, which are from the biphenyl molecule, the L266F residue, and the F354 and F360 phenylalanine residues, associate well with each other (Fig. 1B). Jakoncic et al. (2007) proposed dividing the catalytic pocket of the naphthalene dioxygenase (NDO) from *Sphingomonas* CHY-1 into three regions (distal, central, and proximal), depending on the distance to the mononuclear iron atom. When this proposal is applied to the DK17 *o*-xylene dioxygenase, L266 is in the distal region and F354 and F360 are in the central region. Notably, the two phenylalanine residues in the central region are mostly conserved or have conservative substitutions when compared to related ring ring-hydroxylating dioxygenases including NDOs from *Sphingomonas* CHY-1 and *Pseudomonas* sp. NCIB 9816-4 (Jakoncic et al., 2007). In the case of the DK17 *o*-xylene dioxygenase, however, we found that the seemingly conservative substitution (F354W) led to complete loss of the enzyme activity. This observation can be interpreted to indicate that a too large a hydrophobic side chain does not increase the hydrophobic interactions, but causes a local misfolding of the protein because tryptophan has a bulkier hydrophobic side chain than that of phenylalanine (indole versus phenyl ring). The side chain of F352 (F354 in AkbA1) in the NCIB 9816-4 NDO shields the active site from the more hydrophilic areas behind the phenyl ring. When the phenylalanine was mutated to valine, two additional water molecules were found in the active site, occupying a portion of the cavity created by the mutation (Ferraro et al., 2006). This information leads one to reasonably assume that the dramatically increased activity of the DK17 L266F mutant enzyme against biphenyl may be due to the preventing water from entering the active site, in addition to the higher affinity caused by increased hydrophobic interactions. This hypothesis can also explain why the L266Y enzyme had lower activity against biphenyl than the wild-type enzyme: the hydroxyl side chain of tyrosine may mimic, at least in part, the unfavorable presence of an extra water molecule within the active site.

In conclusion, we combined site-directed mutagenesis, metabolite analysis, and protein modeling to identify hydrophobic interactions and shielding effects against water as important factors in the ability of DK17 *o*-xylene dioxygenase to hydroxylate biphenyl. Although more extensive studies are needed to specifically engineer DK17 *o*-xylene dioxygenase to created tailor-made properties for chemoenzymatic syntheses, our new findings serve as a basis to improve for improving its activity.

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