Crystal Structure of the Terminal Oxygenase Component of Cumene Dioxygenase from *Pseudomonas fluorescens* IP01†

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The crystal structure of the terminal component of the cumene dioxygenase multicomponent enzyme system of *Pseudomonas fluorescens* IP01 (CumDO) was determined at a resolution of 2.2 Å by means of molecular replacement by using the crystal structure of the terminal oxygenase component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 (NphDO). The ligation of the two catalytic centers of CumDO (i.e., the nonheme iron and Rieske [2Fe-2S] centers) and the bridging between them in neighboring catalytic subunits by hydrogen bonds through a single amino acid residue, Asp231, are similar to those of NphDO. An unidentified external ligand, possibly dioxygen, was bound at the active site nonheme iron. The entrance to the active site of CumDO is different from the entrance to the active site of NphDO, as the two loops forming the lid exhibit great deviation. On the basis of the complex structure of NphDO, a biphenyl substrate was modeled in the substrate-binding pocket of CumDO. The residues surrounding the modeled biphenyl molecule include residues that have already been shown to be important for its substrate specificity by a number of engineering studies of biphenyl dioxygenases.

Aromatic hydrocarbons are common contaminants of soil and groundwater (18). One of the most attractive means of removal of these compounds from the environment is the use of microorganisms (34). Dihydroxylation of the aromatic ring by a bacterial aromatic hydrocarbon dioxygenase is a prerequisite for subsequent oxidation of the aromatic nucleus by a ring fission dioxygenase (6). Aromatic hydrocarbon dioxygenases belong to a large family named the Rieske nonheme iron oxygenases (11). Werlen et al. delineated four dioxygenase subfamilies in this large family (the toluene/biphenyl, naphthalene, benzoate, and phthalate subfamilies) based on sequence alignment of the catalytic components (α subunits) (37). The toluene/biphenyl subfamily includes enzymes for the degradation of toluene, benzene, cumene (isopropylbenzene), biphenyl, and polychlorinated biphenyls (PCBs). The naphthalene subfamily consists of enzymes for the degradation of naphthalene and phenanthrene. The Rieske dioxygenases involved in bacterial hydrocarbon degradation comprise multicomponent enzyme systems (36) in which reduced pyridine nucleotide is used as the initial source of two electrons for dioxygen activation. The electrons pass through a flavin cofactor and Rieske [2Fe-2S] centers into the mononuclear iron center of the terminal Rieske nonheme iron dioxygenase component.

The crystal structure of the terminal oxygenase component of naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4 (NphDO) has been reported previously (4, 15, 17), and the structure-function relationship of this enzyme has been

well studied (28). NphDO is an $\alpha_3\beta_3$ hexamer, and each α subunit contains a Rieske [2Fe-2S] cluster and nonheme iron coordinated by His208, His213, and Asp362. The active site iron center of one of the α subunits is directly connected by hydrogen bonds through a single amino acid, Asp205, to the [2Fe-2S] center in a neighboring α subunit, which is the main route of electron transfer (29). A series of NphDO complex structures with indole, oxygen, both indole and oxygen (ternary complex), and naphthalene *cis*-dihydrodiol (product) has been reported, and these structures represent states along a reaction pathway (15). The ternary complex structure with indole and dioxygen bound in a side-on fashion provides the basis for the reaction mechanism of a concerted mode of attack that results in the *cis* specificity of the dihydroxylation.

The terminal components of the biphenyl dioxygenases (BphDOs) involved in the degradation of a highly toxic environmental contaminant, PCB, are also well-studied members of the Rieske nonheme oxygenase family. BphDOs of *Burkholderia cepacia* LB400 (BphDO LB400) and *Pseudomonas pseudoalcaligenes* KF707 (BphDO KF707) have been extensively engineered to improve their capabilities for environmental pollutant degradation by using various techniques, such as random mutagenesis, in vitro DNA shuffling, and subunit or domain exchange (8, 31, 33, 39, 40). For members of the toluene/biphenyl subfamily, however, crystallization of only one enzyme (BphDO from *Burkholderia* sp. strain RHA1 [BphDO RHA1]) has been reported (26). Very recently, the crystal structure of BphDO RHA1 was reported by Furusawa et al. (9).

Cumene is an aromatic hydrocarbon that is intermediate in size between ethylbenzene and biphenyl. *Pseudomonas fluorescens* IP01 has been isolated as a strain that can grow on cumene or toluene as the sole source of carbon. The genes encoding the cumene dioxygenase multicomponent enzyme sys-

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tem (*cumA1*, *cumA2*, *cumA3*, and *cumA4*) and the genes encoding enzymes for subsequent steps (*cumB* to *cumF*) have been cloned (1, 13, 14), and the results indicated that the strain has a *meta*-cleavage degradation pathway very similar to those of biphenyl- and PCB-degrading bacteria. *P. fluorescens* IP01 can also efficiently degrade biphenyl up to the ring fission step catalyzed by a *meta*-cleavage dioxygenase, through the dioxygenation by a cumene dioxygenase. However, the next step (hydrolysis of the *meta*-cleavage product) is blocked because of the strict substrate specificity of the *meta*-cleavage product hydrolase CumD (10, 30). In the multicomponent cumene dioxygenase system, electrons from NADH are thought to be transferred via an iron-sulfur flavoprotein (CumA4) and a Rieske ferredoxin (CumA3) to the terminal component of cumene dioxygenase of *P. fluorescens* IP01 (CumDO) (the α and β subunits are the gene products of *cumA1* and *cumA2*). In this study, we determined the crystal structure of the terminal component of cumene dioxygenase, which exhibits rather high amino acid sequence identity to BphDO LB400 without significant insertions or deletions (74 and 59% for the α and β subunits, respectively). The level of sequence identity between CumDO and BphDO LB400 for the α subunit is slightly higher than that between BphDO RHA1 and BphDO LB400 (69%) and that between CumDO and BphDO RHA1 (67%). The structure provides a good template for modeling of the toluene/biphenyl dioxygenase subfamily in order to discuss the structure-function relationships of these enzymes.

MATERIALS AND METHODS

Expression and purification. The *cumA1* and *cumA2* genes were amplified by PCR by using plasmid pIP103 DNA (30) as a template. The sequences of the forward and reverse primers were ATG GCT AGC ATG AGC TCA ATA AAT AAA G and ATG ATG ATG AGA AGA GCT CAT ATG TAT ATC (the *SacI* sites are underlined), respectively. The amplified DNA was inserted into *SacI*-digested expression vector pUC118. The protein was expressed in *Escherichia coli* BL21(DE3) cells. The CumDO protein was extracted by sonication and purified on DEAE Sephacel (Amersham Biosciences), butyl-Toyopearl (Tosoh), and HiLoad 16/60 Superdex 200 HR (Amersham Biosciences) columns under aerobic conditions.

Absorption spectra. Absorption spectra were measured with a V560 spectrophotometer (JASCO). After a spectrum of the oxidized enzyme as isolated was recorded, a few grains of sodium dithionite were added, and the reduced spectrum was recorded.

Crystallography. Crystallization was conducted by the sitting-drop vapor diffusion method at 25°C; 1 μ l of protein (10 mg of protein per ml in 5 mM Tris-HCl [pH 7.5]) and 1 μ l of a reservoir solution were mixed to form a drop. The best crystallization conditions were obtained with a reservoir solution from a no. 45 PEG/Ion Screen kit (Hampton Research) containing 20% polyethylene glycol 3350 and 0.2 M tris(hydroxymethyl)aminomethane tetrahydrate. Crystals grew in 1 week to maximum dimensions of 0.2 by 0.2 by 0.2 mm. For data collection, selected crystals were transferred to a cryoprotectant solution comprising 10% (vol/vol) polyethylene glycol 400 in the crystallization solution, and then the crystals were flash frozen in a cold nitrogen stream at 100 K. Data were collected with an ADSC Quantum 4R charge-coupled device camera at beamline BL40B2 of SPring-8 (Hyogo, Japan). A total of 150 frames (oscillation width, 0.4°) were collected with an exposure time of 8 s each, and the total time of data collection was 48 min. The collected data were processed with the HKL 2000 program suite (27). Molecular replacement was performed with MOLREP (35) in the CCP4 program suite by using the crystal structure of NphDO (PDB entry 1EG9) as a search model. ARP/wARP was used for automatic model building, starting from the initial phase of the molecular replacement (25). Subsequent refinement was carried out with CNS 1.1 (3). The structure was refined to final *R* and *R*_{free} values of 17.3 and 19.6% at a resolution of 2.2 Å. The data collection and refinement statistics are shown in Table 1. Superimposition of the protein structures was carried out with LSQMAN (20). The figures were generated with Xfit in the

TABLE 1. X-ray crystallography statistics

Parameter	Data
Data collection and processing statistics	
Space group	<i>P</i> 2 ₁ 3
Cell constant (Å)	<i>a</i> = <i>b</i> = <i>c</i> = 147.0
Wavelength (Å)	0.9794
Resolution limit (Å)	20.00–2.20 (2.28–2.20) ^a
No. of observations	836,642
No. of unique reflections	53,921
<i>I</i> / σ (<i>I</i>)	17.7 (6.5)
<i>R</i> _{merge} (%)	11.3 (29.5)
Refinement statistics	
Resolution limits (Å)	19.65–2.20
<i>R</i> factor (%)	17.3
<i>R</i> _{free} (%)	19.6
No. of protein atoms	4,995
No. of ligand atoms	7
No. of water molecules	472
Root mean square deviation from ideal values	
Bond lengths (Å)	0.006
Bond angles (degree)	1.4
Dihedral angles (degree)	23.6

^a The values in parentheses are the values for the last resolution shell.

XtalView program suite (22), MOLSCRIPT (21), Raster3D (23), Pymol (7), and EScript (12).

Docking of substrates. The atoms in the active center of CumDO (the side chain atoms of His234, His240, and Asp388 and nonheme iron) could be superposed on the corresponding atoms of NphDO with a root mean square deviation (RMSD) value of 0.34 Å. The reaction mechanism of CumDO is expected to be the same as that of NphDO, since both enzymes convert aromatic compounds into *cis*-dihydrodiols. These findings suggest that the substrates of CumDO interact with the active center in a manner similar to manner observed for the NphDO-naphthalene complex. We therefore modeled the complex structures of CumDO with its substrates using the crystal structure of the NphDO-naphthalene complex as a template. The following procedure was used for modeling. First, the coordinates of the NphDO-naphthalene complex were translated and rotated to minimize the RMSD value between the active center atoms. Second, the positions of the substrates were calculated by superposition of the aromatic rings of the substrates on one ring of the naphthalene. In this step, the positions of the carbon atoms that underwent *cis*-dihydrogenation in the substrates were matched with those in naphthalene. Finally, the positions were refined by energy minimization by using the SANDER module of the AMBER 6 software package (5) to exclude collision between atoms. During the energy minimization, only the substrate atoms and the protein atoms that were in close contact with the substrate were allowed to move. For biphenyl, 72 conformations which had dihedral angles between the phenyl rings that differed by 5° were examined, and the docking model with the lowest energy (155°) was selected. For cumene, two conformations with isopropyl group dihedral angles of 120° and –60° were compared, and the model with lower energy (120°) was selected. The RMSD values of the final models based on the initial models, calculated for the atoms moved during the energy minimization, were 0.37 and 0.23 Å for the complexes with biphenyl and cumene, respectively.

Accession code of the structure. The coordinates and structure factors have been deposited in the RCSB Protein Data Bank under accession code 1WQL.

RESULTS AND DISCUSSION

Crystallography. The crystal structure of CumDO was determined by molecular replacement by using the crystal structure of NphDO (PDB entry 1EG9) (4) as a search model. In spite of the relatively low sequence identity between CumDO and NphDO (32 and 23% for the α and β subunits, respectively), the molecular replacement solution gave a good initial phase for subsequent automated chain tracing and crystallographic refinement. The final model of the CumDO crystal structure contained residues Asn19 to Asp146 and Cys152 to Ser459 of the α subunit, residues Asp5 to Phe186 of the β

FIG. 1. Ribbon representations of αβ heterodimers of CumDO (a) and NphDO (b). The α and β subunits are grey and yellow, respectively. Iron atoms and sulfur atoms are represented by dark red and yellow spheres, respectively. Loop 1 (a) and loop 2 (b) are represented by blue and green tubes for CumDO and NphDO, respectively. The arrow indicates the possible entrance for a substrate into the CumDO active site.

subunit, one Rieske [2Fe-2S] cluster, a nonheme iron atom, a dioxygen molecule (putative) in the α subunit, and 472 water molecules. A disulfide bond was formed between Cys145 and Cys152, but the residues between these residues were disordered. The αβ heterodimers in the asymmetric unit were related by a crystallographic threefold axis to generate an α₃β₃ heterohexamer. The quaternary, tertiary (Fig. 1), and secondary structures of CumDO are similar to those of NphDO (the RMSD was 1.4 Å for 372 residues of the α subunit, and the RMSD was 1.3 Å for 155 residues of the β subunit), except for several regions, including the two loops at the entrance of the substrate-binding pocket (see below).

Catalytic components. Nonheme iron in the active site was coordinated by His234, His240, and Asp388 (Fig. 2a). Asp388 underwent monodentate coordination, which was evident from both the greater distance of the OD1 atom from Fe (see Table S1 in the supplemental material) and the electron density map. In NphDO, however, the corresponding Asp362 residue has been reported to undergo bidentate coordination (4, 17). At the positions corresponding to the sites of dioxygen in the binary and ternary complexes of NphDO, electron density corresponding to two light atoms, such as oxygen, was observed (Fig. 2a and c). Although the chemical nature of these external atoms was not clear, we tentatively added a dioxygen molecule to the peak in the final refined model. The O-O distance of the putative dioxygen molecule was refined to be 1.46 Å under the restraint of 1.45 Å, and the temperature factors of the oxygen atoms were refined to be 25.8 Å² (O1) and 29.4 Å² (O2). The O1 atom was liganded to Fe, whereas the O2 atom was not (see Table S1 in the supplemental material). On the other hand, in the binary complex structure of NDO with dioxygen (PDB code 1O7 M), the distances of oxygen atoms from Fe are comparable (2.1 and 2.3 Å). There is another possibility, that two water molecules or ions with partial

occupancies are bound. When we placed one oxygen atom (water or hydroxide) into each position and refined the structure, the $F_o - F_c$ map obtained still exhibited excess residual electron density besides the oxygen atom (Fig. 2c, red and blue maps). The distance between these oxygen atoms was 1.36 Å. In the crystal structure of BphDO RHA1, a similar electron density peak for an uncertain external ligand(s) was observed at the nonheme iron site (9). Therefore, the geometry of the nonheme iron in CumDO can be described as distorted tetrahedral coordination (His, His, Asp, and an external ligand atom), which looks very similar to the geometry reported for BphDO RHA1. This structural feature may represent an oxidized or inactive state of Rieske nonheme iron dioxygenases, because the CumDO protein sample was purified in an inactive state under aerobic conditions. The UV-visible absorption spectrum of the CumDO protein sample (see Fig. S1 in the supplemental material) had a large peak at 280 nm and a smaller peak at 450 nm. The smaller peak was possibly due to the Rieske [2Fe-2S] cluster, the level of which was decreased upon addition of sodium dithionite. This suggests that the Rieske [2Fe-2S] cluster of the purified sample had been oxidized and then was reduced with sodium dithionite. However, the oxidation state of the nonheme iron was not certain based on the UV-visible absorption spectrum, and reduction in the strong X-ray beam from the synchrotron radiation source (16) must have occurred to some extent. Because the electron density peak for nonheme iron atom was clearly observed, the inactivation of the enzyme was not due to loss of the iron (38). Furusawa et al. suggested that the oxidation of the nonheme iron and the Rieske cluster caused the inactivation of BphDO RHA1. Because the structural features of the catalytic center of CumDO are very similar to those of BphDO RHA1, this center also seems to represent an oxidized or inactive state. As discussed by Furusawa et al. (9), it is possible

FIG. 2. Electron density maps. (a) Nonheme iron center with a $2F_o - F_c$ map (1.5σ) (blue). Symmetry-related molecules are represented by red sticks. Water molecules are represented by red spheres. (b) Rieske [2Fe-2S] center with a $2F_o - F_c$ map (1.5σ). (c) External ligands for the nonheme iron. The green map is an $F_o - F_c$ omit map (6σ) of both ligand atoms. The red and blue maps are $F_o - F_c$ maps (6σ) for ligand atoms (cyan spheres).

that the external ligand is specific for the oxidized form at the nonheme iron and causes the inactivation of the enzyme. Further analyses are required to resolve the potential influence of the oxidation state of the catalytic components on the activity of Rieske nonheme iron dioxxygenases.

In the Rieske [2Fe-2S] center, Fe1 is coordinated by Cys101 and Cys121, while Fe2 is coordinated by His103 and His124 (Fig. 2b). The two centers in the symmetry-related α subunits are connected through hydrogen bonds from His234 to His124 bridged by a single amino acid, Asp231. Therefore, ligation of

FIG. 3. Amino acid sequence alignment of parts of the α subunit of CumDO, BphDOs, and NphDO. The numbers above the alignment are the numbers in the CumDO sequence. The secondary structures and their designations are indicated above the CumDO sequence and below the NphDO sequence. Completely conserved residues are indicated by boldface type. Nonheme iron ligand residues, conserved Asn/Gln residues, and the bridging Asp residue are highlighted. Residues forming the inner surface of the substrate-binding pocket of CumDO are indicated by triangles. Loops 1 and 2 of CumDO and NphDO and regions I to IV of BphDO LB400 are enclosed in boxes (see text).

the Rieske [2Fe-2S] center and the bridging of the two centers are similar to the ligation and bridging in NphDO, indicating that the basic reaction mechanism of the naphthalene and toluene/biphenyl subfamilies is conserved.

Asn201 of NphDO is located close to the Fe atom, but the distance is too great (3.8 Å) for this residue to be an iron ligand (17). The asparagine residue is also thought to deliver protons if the reaction is carried out through protonated reactive peroxide (15). However, the corresponding residue is Gln in the toluene/biphenyl subfamily (Fig. 3). The side chain of Gln227 of CumDO at this position is longer than that of asparagine. We could not confidently determine the direction of the amide group of Gln227, but one atom of the amide group (possibly nitrogen) is located 3.4 Å from Fe. Another atom of the amide group (possibly oxygen) forms a strong hydrogen bond (2.6 Å) with the hydroxyl group of Tyr123 in the symmetry-related α subunit. The distance between the corresponding pair of atoms (Asn201 OD and Tyr103 OH) in NphDO is 3.2 Å. The side chain amide group of Gln227 in CumDO is located far from bound dioxygen and does not interact with the bound dioxygen. Therefore, a certain conformational change is required if the glutamine residue is involved in iron ligation and/or proton delivery.

Active site pocket and substrate specificity. Two loops of NphDO (loop 1, Ile223 to Leu240; and loop 2, Leu253 to Leu265) (Fig. 1b, green tubes) are thought to act as lids covering the channel to the active site (17). However, the corre-

sponding loops of CumDO (loop 1, Leu248 to Asn264; and loop 2, Phe278 to Gly290) (Fig. 1a, blue tubes) differ significantly. The average temperature factors of the main chain atoms in these regions (31.6 and 17.9 Å² for loops 1 and 2 of NphDO and 36.9 and 24.6 Å² for loops 1 and 2 of CumDO) are relatively high compared with those of the whole α subunits (14.1 and 21.0 Å² for NphDO and CumDO, respectively). In particular, loop 1 of CumDO is greatly shifted to run parallel with loop 2, opening a channel to the active site from the neighboring position (Fig. 1a). The C-terminal part of loop 2 forms an α -helix and makes an acute-angle bend at a conserved glycine residue (Gly290) to the following helix region. Mobile helix α 9 of BphDO RHA1, which contains residues that shift upon substrate binding, corresponds to the C-terminal part of loop 2 (9). The two loops are also involved in formation of the active site pocket, as discussed below.

In order to discuss the substrate specificity of the toluene/biphenyl subfamily on the basis of the results for BphDOs, cumene and biphenyl molecules were modeled in the refined structure of CumDO. Figure 4a and b shows the molecular surface of CumDO, along with the modeled substrates. The side chains of 14 residues (Fig. 3) contribute to the inner surface of the substrate-binding pocket. One of these residues, Met232, is notable and contributes to the more hydrophobic surface of CumDO compared with the surface of NphDO (Fig. 4c). The pocket of CumDO appears to be large enough to

FIG. 4. Molecular surface at the substrate-binding pocket of CumDO with modeled biphenyl (a) and cumene (b) and molecular surface at the substrate-binding pocket of NphDO complexed with indole (c). The atoms at the surface are indicated by different colors (red, oxygen; blue, nitrogen; green, sulfur).

accommodate the biphenyl substrate, and the entrance of the pocket is totally different from that of NphDO. It has been reported that the substrate-binding pocket of BphDO RHA1 undergoes significant conformational changes upon substrate

(biphenyl) binding and that the two rings of the bound biphenyl molecule are significantly skewed (9). Although the modeled biphenyl molecule in CumDO is not greatly skewed (the angle between the planes is 25°), it is located at a position similar to the position in the BphDO RHA1 biphenyl complex, as described below.

Figure 5a shows superimposition of the CumDO with modeled biphenyl and the ternary complex of NphDO with indole and oxygen (PDB code 1O7N). Residues of CumDO whose side chain atoms are located within $4.0 \text{ \AA}$ of the modeled biphenyl are shown in Fig. 5a (blue ball and stick model), as are other residues corresponding to residues of BphDOs that have been shown to be important for substrate specificity, as described below (cyan). Mondello et al. identified four regions of BphDO LB400 and BphDO KF707 (regions I, II, III, and IV) (Fig. 3 and 5) in which specific sequences were consistently associated with either broad or narrow PCB substrate specificity (24). Some individual mutations within region III alone improve PCB degradation activity, especially with di-*para*-substituted congeners. As shown in Fig. 5a, three of the four regions (regions II, III, and IV) are located near the modeled biphenyl, indicating that the modeling study was properly conducted. Region I is located between the two iron-ligating histidines and differs significantly from the corresponding region of NphDO. Other lines of experimental evidence are consistent with the assumption concerning the binding site in the CumDO structure. Kimura et al. (19) and Suenaga et al. (31, 32) showed that a mutation at Thr376 (Thr377 in CumDO) in BphDO KF707 resulted in an ability to degrade PCB congeners. Bruhlmann and Chen obtained some evolved BphDOs which could recognize both *ortho*- and *para*-substituted PCBs by DNA shuffling between LB400 and KF707 and found that all variants contained Thr335Ala and Phe336Ile substitutions (Gly335 and Ile336 in CumDO) (2). Zielinski et al. reported that mutations at Met231, Phe378, and Phe384 (Met232, Phe378, and Tyr384 in CumDO) greatly altered the regioselectivity of substrate dioxygenation of BphDO LB400 (40). For NphDO, Phe352 (Phe378 in CumDO) has been shown to be a crucial residue for regio- and enantioselectivity (28).

The large number of engineering studies of BphDOs described above involved various techniques for gene evolution, but the resultant mutations of the evolved genes are rather concentrated in specific regions in the three-dimensional space, as found in this study. Zielinski et al. (40) and Suenaga et al. (33) used the NphDO structure as the template for homology modeling of BphDO LB400 and BphDO KF707 and obtained fruitful results. However, some regions or residues which appear to be important for the substrate specificities of BphDOs but have not been thoroughly tested for engineering studies yet, such as Ile288 in loop 2, remain to be examined. Large deviations in loops 1 and 2 from the template structure used for modeling (NphDO) seem to have impaired the quality of modeling. The combination of gene evolution techniques and rational designs based on crystal structures should accelerate the engineering of BphDOs in the future.

Figure 5b shows superimposition of CumDO with the BphDO RHA1 structure complexed with biphenyl (PDB code 1ULJ; the RMSD for 411 residues of the α subunit was $0.76 \text{ \AA}$), which was reported very recently (9). The modeled biphenyl in CumDO (Fig. 5b) is located far from the nonheme iron,

a

b

FIG. 5. Superimposition of CumDO and related structures at the active site. (a) Superimposition of CumDO (blue) and NphDO (green). The modeled biphenyl (yellow) and putative dioxygen (red) of CumDO, as well as indole (orange) and dioxygen (green) in NphDO, are shown as ball and stick models with different colors of sticks. The side chains of catalytically important residues of CumDO and NphDO and the side chains of the residues forming the substrate-binding pocket of CumDO are shown as ball and stick models. Regions I to IV, which are involved in the substrate specificity of BphDO LB400, are shown in red, and loop 1 and loop 2 are represented by thick tubes. The blue and green arrows indicate possible entrances to the pockets of CumDO and NphDO, respectively. (b) Superimposition of CumDO (blue) and BphDO RHA1 complexed with biphenyl (PDB code 1HIJ) (green). The modeled biphenyl (yellow) and putative dioxygen molecule (red) of CumDO, as well as the biphenyl (orange) in BphDO RHA1, are represented by sticks that are different colors. The side chains of the residues around the biphenyl rings are shown as ball and stick models. Phe278 and Tyr384 in CumDO are replaced by Tyr and Phe residues in BphDO RHA1, respectively. Other details are the same as those described above for panel a.

compared with the biphenyl molecule observed in the complex structure of BphDO RHA1 (Fig. 5b), probably because we performed the docking study using the CumDO structure with a putative dioxygen molecule. However, the approximate positions of these biphenyl molecules are virtually same. The residues around the biphenyl molecule are very similar in the two structures, except in the following four regions. (i) The conformation of the side chain of Met232 is different from that of Met222 in BphDO RHA1. (ii) Phe278 and Tyr384 in CumDO are replaced by Tyr and Phe residues in BphDO RHA1, respectively. (iii) Part of loop 1 (from position 238 to position 249) is not observed in the BphDO RHA1 structure, probably due to disorder. (iv) The main chain trace of loop 2 containing Leu284 and Ile288 is significantly different. It is not clear if these differences influence the activity of CumDO and BphDO RHA1, because a detailed study of the substrate specificity of CumDO has not been conducted yet. However, the superb resemblance at the active site pockets of CumDO and BphDO is consistent with the finding that CumDO can effectively act on the biphenyl substrate, as well as cumene (1).

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REFERENCES

- Aoki, H., T. Kimura, H. Habe, H. Yamane, T. Kodama, and T. Omori. 1996. Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-7-methylocta-2,4-dienoic acid in *Pseudomonas fluorescens* IP01. *J. Ferment. Bioeng.* **81**:187–196.
- Brühlmann, F., and W. Chen. 1999. Tuning biphenyl dioxygenase for extended substrate specificity. *Biotechnol. Bioeng.* **63**:544–551.
- Brunger, A. T., P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson, and G. L. Warren. 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. Sect. D* **54**:905–921.
- Carredano, E., A. Karlsson, B. Kauppi, D. Choudhury, R. E. Parales, J. V. Parales, K. Lee, D. T. Gibson, H. Eklund, and S. Ramaswamy. 2000. Substrate binding site of naphthalene 1,2-dioxygenase: functional implications of indole binding. *J. Mol. Biol.* **296**:701–712.
- Case, D. A., D. A. Pearlman, J. W. Caldwell, T. E. Cheatham III, W. S. Ross, C. L. Simmerling, T. A. Darden, K. M. Merz, R. V. Stanton, A. L. Cheng, J. J. Vincent, M. Crowley, V. Tsui, R. J. Radmer, Y. Duan, J. Pitera, I. Massova, G. L. Seibel, U. C. Singh, P. K. Weiner, and P. A. Kollman. 1999. AMBER 6. University of California, San Francisco.
- Dagley, S. 1986. Biochemistry of aromatic hydrocarbon degradation in *Pseudomonads*, p. 527–555. In J. R. Sokatch and L. N. Ornston (ed.), *The bacteria*, vol. 10. Academic Press, New York, N.Y.
- DeLano, W. L. 2002. The PyMOL molecular graphics system. DeLano Scientific, San Carlos, Calif.
- Furukawa, K. 2000. Engineering dioxygenases for efficient degradation of environmental pollutants. *Curr. Opin. Biotechnol.* **11**:244–249.
- Furusawa, Y., V. Nagarajan, M. Tanokura, E. Masai, M. Fukuda, and T. Senda. 2004. Crystal structure of the terminal oxygenase component of biphenyl dioxygenase derived from *Rhodococcus* sp. strain RHA1. *J. Mol. Biol.* **342**:1041–1052.
- Fushinobu, S., T. Saku, M. Hidaka, S. Y. Jun, H. Nojiri, H. Yamane, H. Shoun, T. Omori, and T. Wakagi. 2002. Crystal structures of a meta-cleavage product hydrolase from *Pseudomonas fluorescens* IP01 (CumD) complexed with cleavage products. *Protein Sci.* **11**:2184–2195.
- Gibson, D. T., and R. E. Parales. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotechnol.* **11**:236–243.
- Gouet, P., E. Courcelle, D. I. Stuart, and F. Metoz. 1999. ESPript: multiple sequence alignments in PostScript. *Bioinformatics* **15**:305–308.
- Habe, H., K. Kasuga, H. Nojiri, H. Yamane, and T. Omori. 1996. Analysis of cumene (isopropylbenzene) degradation genes from *Pseudomonas fluorescens* IP01. *Appl. Environ. Microbiol.* **62**:4471–4477.
- Habe, H., T. Kimura, H. Nojiri, H. Yamane, and T. Omori. 1996. Cloning and nucleotide sequences of the genes involved in the meta-cleavage pathway of cumene degradation in *Pseudomonas fluorescens* IP01. *J. Ferment. Bioeng.* **81**:247–254.
- Karlsson, A., J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund, and S. Ramaswamy. 2003. Crystal structure of naphthalene dioxygenase: side-on binding of dioxygen to iron. *Science* **299**:1039–1042.
- Karlsson, A., J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund, and S. Ramaswamy. 2000. The reduction of the Rieske iron-sulfur cluster in naphthalene dioxygenase by X-rays. *J. Inorg. Biochem.* **78**:83–87.
- Kauppi, B., K. Lee, E. Carredano, R. E. Parales, D. T. Gibson, H. Eklund, and S. Ramaswamy. 1998. Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. *Structure* **6**:571–586.
- Keith, L. H., and W. A. Telliard. 1979. Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13**:416–423.
- Kimura, N., A. Nishi, M. Goto, and K. Furukawa. 1997. Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. *J. Bacteriol.* **179**:3936–3943.
- Kleywegt, G. J. 1996. Use of non-crystallographic symmetry in protein structure refinement. *Acta Crystallogr. Sect. D* **52**:842–857.
- Kraulis, P. J. 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**:946–950.
- McRee, D. E. 1999. XtalView/Xfit—a versatile program for manipulating atomic coordinates and electron density. *J. Struct. Biol.* **125**:156–165.
- Merritt, E. A., and D. J. Bacon. 1997. Raster3D: photorealistic molecular graphics. *Methods Enzymol.* **277**:505–524.
- Mondello, F. J., M. P. Turcich, J. H. Lobos, and B. D. Erickson. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. *Appl. Environ. Microbiol.* **63**:3096–3103.
- Morris, R. J., A. Perrakis, and V. S. Lamzin. 2002. ARP/wARP's model-building algorithms. I. The main chain. *Acta Crystallogr. Sect. D* **58**:968–975.
- Nagarajan, V., N. Sakurai, M. Kubota, T. Nonaka, H. Nagumo, H. Takeda, T. Nishizaki, E. Masai, M. Fukuda, Y. Mitsui, and T. Senda. 2003. Crystallization of the terminal oxygenase component of biphenyl dioxygenase derived from *Rhodococcus* sp. strain RHA1. *Protein Pept. Lett.* **10**:412–417.
- Otwinowski, Z., and W. Minor. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**:307–326.
- Parales, R. E. 2003. The role of active-site residues in naphthalene dioxygenase. *J. Ind. Microbiol. Biotechnol.* **30**:271–278.
- Parales, R. E., J. V. Parales, and D. T. Gibson. 1999. Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity. *J. Bacteriol.* **181**:1831–1837.
- Saku, T., S. Fushinobu, S.-Y. Jun, N. Ikeda, H. Nojiri, H. Yamane, T. Omori, and T. Wakagi. 2002. Purification, characterization, and steady-state kinetics of a meta-cleavage compound hydrolase from *Pseudomonas fluorescens* IP01. *J. Biosci. Bioeng.* **93**:567–574.
- Suenaga, H., M. Mitsuoka, Y. Ura, T. Watanabe, and K. Furukawa. 2001. Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene, and alkylbenzenes. *J. Bacteriol.* **183**:5441–5444.
- Suenaga, H., A. Nishi, T. Watanabe, M. Sakai, and K. Furukawa. 1999. Engineering a hybrid pseudomonad to acquire 3,4-dioxygenase activity for polychlorinated biphenyls. *J. Biosci. Bioeng.* **87**:430–435.
- Suenaga, H., T. Watanabe, M. Sato, Ngadiman, and K. Furukawa. 2002. Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. *J. Bacteriol.* **184**:3682–3688.
- Timmis, K. N., and D. H. Pieper. 1999. Bacteria designed for bioremediation. *Trends Biotechnol.* **17**:200–204.
- Vagin, A., and A. Teplyakov. 1997. MOLREP: an automated program for molecular replacement. *J. Appl. Crystallogr.* **30**:1022–1025.
- Wackett, L. P. 2002. Mechanism and applications of Rieske non-heme iron dioxygenases. *Enzyme Microb. Technol.* **31**:577–587.
- Werlen, C., H. P. Kohler, and J. R. van der Meer. 1996. The broad substrate chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. *J. Biol. Chem.* **271**:4009–4016.
- Wolfe, M. D., D. J. Altier, A. Stubna, C. V. Popescu, E. Munck, and J. D. Lipscomb. 2002. Benzoate 1,2-dioxygenase from *Pseudomonas putida*: single turnover kinetics and regulation of a two-component Rieske dioxygenase. *Biochemistry* **41**:9611–9626.
- Zielinski, M., S. Backhaus, and B. Hofer. 2002. The principal determinants for the structure of the substrate-binding pocket are located within a central core of a biphenyl dioxygenase alpha subunit. *Microbiology* **148**:2439–2448.
- Zielinski, M., S. Kahl, H. J. Hecht, and B. Hofer. 2003. Pinpointing biphenyl dioxygenase residues that are crucial for substrate interaction. *J. Bacteriol.* **185**:6976–6980.