Crystal Structure of the Oxygenase Component (HpaB) of the 4-Hydroxyphenylacetate 3-Monoxygenase from Thermus thermophilus HB8*

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The 4-hydroxyphenylacetate (4HPA) 3-monoxygenase is involved in the initial step of the 4HPA degradation pathway and catalyzes 4HPA hydroxylation to 3,4-dihydroxyphenylacetate. This enzyme consists of two components, an oxygenase (HpaB) and a reductase (HpaC). To understand the structural basis of the catalytic mechanism of HpaB, crystal structures of HpaB from Thermus thermophilus HB8 were determined in three states: a ligand-free form, a binary complex with FAD, and a ternary complex with FAD and 4HPA. Structural analysis revealed that the binding and dissociation of flavin are accompanied by conformational changes of the loop between β5 and β6 and of the loop between β8 and β9, leading to preformation of part of the substrate-binding site (Ser-197 and Thr-198). The latter loop further changes its conformation upon binding of 4HPA and obstructs the active site from the bulk solvent. Arg-100 is located adjacent to the putative oxygen-binding site and may be involved in the formation and stabilization of the C4a-hydroperoxyflavin intermediate.

The 4-hydroxyphenylacetate (4HPA)2 3-monoxygenase catalyzes hydroxylation of 4HPA to 3,4-dihydroxyphenylacetate (DHPA). This enzyme is involved in the initial step of the degradation pathway of 4HPA. It uses molecular oxygen and reduced flavin for hydroxylation and NAD(P)H for flavin reduction. Thus, the enzymatic reaction is separated into two steps, the reduction of flavin to generate two reducing equivalents using NAD(P)H as an electron donor and the hydroxylation of substrates using molecular oxygen and reduced flavin. This enzyme has been isolated from Escherichia coli (1–4), Pseudomonas putida (5, 6), Klebsiella pneumoniae (7), and Acinetobacter baumannii (8, 9). The small component of these enzymes is the flavin reductase, which generates reduced flavin for the oxygenase component to oxygenate substrates. Hydroxylation activity is dependent on the presence of the reductase component (1–9). Galán et al. (10) have classified these enzymes into the two-component flavin-diffusible monoxygenase (TC-FDM) family (10). The TC-FDM family includes monoxygenases targeting various substrates such as 4HPA, chlorophenol (11), styrene (12), phenol (13), p-nitrophenol (14), nitrotriacetate (15), EDTA (16), and aliphatic sulfonate (17). Amino acid sequences of reductase components are relatively similar to each other, whereas those of oxygenase components differ, which may reflect substrate specificity for either aromatic or non-aromatic compounds (14). Recent studies indicate that members of the TC-FDM family that act on aromatic compounds can be further divided into two groups. One group includes an enzyme from A. baumannii (and probably one from P. putida), and the other includes the remaining members (the E. coli enzyme is representative of this group). The reductase component (C2) from A. baumannii is larger than E. coli-type reductases of the TC-FDM family and is stimulated by the presence of the substrate (18). In contrast, E. coli-type reductases are not affected by the presence of their substrate. The oxygenase component (C1) from A. baumannii shows low identity with E. coli-type oxygenases (9). Moreover, C2 reacts with FADH2, FMNH2, and riboflavin during the hydroxylation reaction (19), whereas E. coli-type oxygenases selectively utilize either FADH2 or FMNH2 (20).

Hydroxylation of aromatic compounds by single-component flavoenzyme hydroxylases, including p-hydroxybenzoate hydroxylase (21–23), 3-hydroxybenzoate hydroxylase (24), and phenol hydroxylase (25) has been reported. The catalytic mechanisms of p-hydroxybenzoate hydroxylase have been investigated thoroughly by many structural and kinetic studies (Refs. 21–23 and references therein). This enzymatic reaction can be divided into reduction and oxidation. In the reductive reaction the enzyme changes its structure from an open conformation to a closed (or the in) form upon binding of substrate. Subsequently, NADPH binds to the enzyme, and the isoalloxazine ring of FAD swings toward the protein surface (the out conformation), allowing electron transfer from NADPH to FAD. In the oxidative reaction reduced FAD moves back to the in con-

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The atomic coordinates and structure factors (code 2YYG, 2YYI, and 2YYJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: 4HPA, 4-hydroxyphenylacetate; HpaB, the oxygenase component of 4-hydroxyphenylacetate 3-monoxygenase; HpaC, the reductase component of 4-hydroxyphenylacetate 3-monoxygenase; DHPA, 3,4-dihydroxyphenylacetate; TC-FDM, two-component flavin-diffusible monoxygenase; r.m.s.d., root mean square deviations; C2, oxygenase component of 4-hydroxyphenylacetate 3-hydroxylase from A. baumannii.
Crystal Structure of HpaB from Thermus thermophilus

The amino acid sequences of the HpaB (54.3 kDa) and HpaC (16.1 kDa) proteins from Thermus thermophilus HB8 have identities of 30 and 29%, respectively, with the corresponding proteins from E. coli. The unique feature of these enzymes is that they can bind and release flavin. We reported previously the crystal structure of HpaC, showing that no conformational changes occur upon FAD binding (28). The affinity of HpaC for FAD can be attributed to the interaction between the AMP moiety of FAD and the non-conserved loop region (Gly-83-Gly-94) of HpaC.

In this research, to understand the structural basis for the catalytic mechanism, we determined the crystal structures of HpaB from T. thermophilus HB8 in three states; that is, a ligand-free form, a FAD complex, and a FAD-substrate complex. This is the first structural determination for the type oxygenase components in the TC-FDM family. The unique feature of these enzymes is that they can bind and release flavin. We reported previously the crystal structure of HpaC, showing that no conformational changes occur upon FAD binding (28). The affinity of HpaC for FAD can be attributed to the interaction between the AMP moiety of FAD and the non-conserved loop region (Gly-83-Gly-94) of HpaC.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—The purification, crystallization, and initial phase determination of HpaB has been described elsewhere (29). The structure of HpaB was determined using the multiwavelength anomalous diffraction method.
method with a platinum derivative (29). As the model was being built, three residues were found to be mutated due to a PCR error (T198I, A276G, and R466H). The expression plasmid was reconstructed, and purification of wild-type HpaB was performed in the same manner as was used to purify the mutated protein. Crystals of wild-type HpaB were prepared by mixing 4 μl of a solution containing 5 mg/ml protein and 1 mM dithiothreitol with an equal volume of a reservoir solution containing 1.5 M ammonium sulfate, 0.1 M Tris-HCl (pH 8.5), and 25% (v/v) glycerol. Crystals appeared within a few minutes and reached a final dimension of 0.25 × 0.4 × 0.2 mm after 1–3 days. Crystals of the HpaB\textsubscript{FAD} complex were obtained by soaking crystals of the ligand-free form into a reservoir solution containing 5 mM FAD for 30 min. For crystals of the HpaB\textsubscript{FAD-4HPA} complex, crystals obtained in the presence of 5 mM 4HPA were soaked in 5 mM FAD for 210 min.

Data Collection and Structural Determination—Diffraction data for wild-type HpaB, the HpaB-FAD complex, and the HpaB-FAD-4HPA complex were collected under cryogenic conditions using a Quantum 210 CCD detector (ADSC) at beamline BL44B2 at SPring-8, Harima, Japan (30). The data were indexed, integrated, and scaled using the HKL2000 program (31) and were further processed using the CCP4 suite (32). Data collection statistics are summarized in Table 1.

Models of wild-type HpaB, the HpaB-FAD complex, and the HpaB-FAD-4HPA complex were built using the ARP/wARP program (33), with the model of mutated HpaB serving as the initial model. Further model building and refinements were carried out using the programs XtalView (34) and CNS (35). Model analysis was carried out with PROCHECK (36). The refinement statistics are summarized in Table 2.

RESULTS AND DISCUSSION

Quality of the Models—The crystal structures of unliganded HpaB, the HpaB-FAD complex, and the HpaB-FAD-4HPA complex were determined at resolutions of 2.00, 1.66, and 1.66 Å, respectively. No electron density was observed for the one N-terminal residue (Met-1) and four C-terminal residues (Glu-478—Ala-481). In addition, in the model of unliganded HpaB and that of the HpaB\textsubscript{FAD} complex, the Arg-153—Gln-158 and Ser-197–Thr-198 residues, respectively, were not clearly defined in electron densities and were omitted from the final models. A Ramachandran plot for each model showed that more than 91% of the non-glycine residues fall into the most favored regions and that Met-246 lies in disallowed regions. This residue is anterior to the conserved Asp-247, which forms hydrogen bonds with Thr-185 and two arginine residues (Arg-225 and Arg-433). The unusual main-chain conformation of Met-246 is probably required for the formation of the hydrogen bond network that stabilizes the C4a-hydroperoxyflavin intermediate. In addition, in the model of unliganded HpaB, Thr-145 also lies in disallowed regions. Thr-145 is located at the starting position of the loop between β5 and β6. Thr-145 and Leu-144 moved significantly upon binding of FAD, and the conformational change of the main chain of this dipeptide affected the conformations of Ser-197 and Thr-198 (see below). Thus, the unusual main chain
Crystal Structure of HpaB from Thermus thermophilus
conformation of Thr-145 may contribute to preformation of the substrate-binding site. The electron densities of Thr-145 and Met-246 were clearly defined (data not shown).

Structure of HpaB—The crystal structure of HpaB is shown in Fig. 1. HpaB is a tetrameric molecule and is arranged as a dimer of dimers (Fig. 1A). The HpaB monomer consists of three domains, the N-terminal (Ala-2—Leu-138), middle (Ala-139—Gly-266), and C-terminal (Asn-267—Tyr-456) domains, with an additional C-terminal α-helical tail (Asn-457—Ala-481) (Figs. 1B and 2). The N- and C-terminal domains consist mainly of antiparallel α-helices, and the middle domain is packed with two β-sheets. One β-sheet consists of β5, β8, β9, and β12, and the other consists of β1, β2, β6, β7, β10, and β11. Two of the C-terminal α-helical tails protrude from the dimer and extend to the other dimer, lying on its surface. There is a groove between the three domains that serves as the binding sites of FADH₂ and a substrate.

The overall structure of the HpaB-FAD complex is similar to that of the ligand-free form, with a r.m.s.d. of 0.865 Å for 468 Cα atoms (Ala-2—Ala-152, Pro-159—Pro-196, and Leu-199—Phe-478). However, conformational differences are observed in the loop between β5 and β6 (loop β5–β6) and in the loop between β8 and β9 (loop β8–β9) (Figs. 3A and B). In addition, the electron densities of residues Arg-153—Gln-158 in loop β5–β6 that were not seen in the ligand-free model are now visualized. The Pro-155—Gln-158 region in loop β5–β6 forms a 3₁₀-helix. The conformational change of loop β5–β6 also

**FIGURE 1.** Amino acid sequence alignment of several oxygenase components belonging to the TC-FDM family and 4-hydroxybutyryl-CoA dehydratase whose structure is most similar to that of HpaB. Secondary structures in HpaB are indicated above the alignment. α-helices and β-strands are indicated by cylinders and arrows, respectively, and the colors correspond to those in Fig. 1B. HpaB shows 21–32% homology with other enzymes. Entirely conserved residues among these proteins and the TC-FDM family are indicated by red and blue boxes, respectively, and other conserved residues are indicated by open boxes. Triangles indicate the residues that are involved in substrate binding. HpaB, HpaB from T. thermophilus HB8 (gi:55772342); HpaB_E.coli, HpaB from E. coli (gi:6983686); PheA1, phenol 2-hydroxylase component A from Geobacillus thermoglucosidasius (gi:7672524); HpaA, oxygenase component of 4-hydroxyphenylacetate 3-hydroxylase from K. pneumoniae (gi:974145); Tfd, chlorophenol 4-monoxygenase component 2 from Burkholderia cepacia (gi:21954029); HsdA, chlorophenol 4-hydroxylase from Ralstonia pickettii (gi:847716); 4-BUDH, 4-hydroxybutyryl-CoA dehydrogenase from C. aminobutyricum (gi:58176904).
FIGURE 4. The structure of the HpaB-FAD complex. A, the $F_o - F_c$ omit map for FAD contoured at the 3.5 $\sigma$ is represented in light blue. B, stereoview of the FAD-binding site showing amino acid residues interacting with FAD. Carbon atoms of FAD, residues, and residues from the other monomer are shown in yellow, white, and light blue, respectively. Oxygen, nitrogen, and phosphorus atoms are shown in red, blue, and green, respectively. Water molecules are shown in magenta. Black dotted lines indicate hydrogen bonds. C, surface representation of the HpaB-FAD complex using the same color scheme as in Fig. 1A. Loop $\beta_5-\beta_6$ and loop $\beta_8-\beta_9$ (all from monomer A) are shown in cyan and orange, respectively. Labels show each monomer, and FAD is shown as a stick model. Water molecules are shown as white spheres.
causes a change in the length of β5. In unliganded HpaB, β5 was assigned from Thr-140 to Leu-144, whereas in the HpaB-FAD complex, it was assigned from Thr-140 to His-142. An important observation is that Leu-144, which occupies part of the FAD binding site in the ligand-free form, is pushed away upon the binding of FAD (Fig. 3C). The movement of Leu-144 in turn affects the conformation of the region around residues Ser-197 and Thr-198 as well as the remaining region of loop β8–β9, resulting in the preformation of the substrate-binding site. Data for a crystal of HpaB obtained in the presence of 4HPA indicated no electron density for 4HPA, and the structure of HpaB was essentially identical to that of unliganded HpaB, indicating that the binding of FAD(H₂) was essential for the preformation of the substrate-binding site (data not shown).

The structure of the HpaB-FAD-4HPA complex is similar to those of unliganded HpaB and the HpaB-FAD complex, with r.m.s.d. values of 0.865 Å for 470 Ca atoms (Ala-2—Ala-152 and Pro-159—Phe-478) and 0.694 Å for 475 Ca atoms (Ala-2—Pro-196 and Leu-199—Phe-478), respectively (Fig. 3A). Differences among the three structures are observed in loop β8–β9 (Phe-195—Tyr-207) (Fig. 3B). The conformation of loop β8–β9 was further changed compared with that of the HpaB-FAD structure, establishing Ser-197 and Thr-198 as binding sites for the carboxyl group of 4HPA. The main chain of Thr-198 in loop β8–β9 exhibits the largest shifts (7.6 Å) compared with that of the ligand-free form. This conformational change acts to obstruct the catalytic site from the bulk solvent during hydroxylation (see Fig. 5C). Therefore, molecular oxygen could not access the catalytic site after the substrate binding.

FAD Binding—The electron density of FAD in the HpaB-FAD complex is clearly observed in the groove (Fig. 4A). FAD has an extended conformation and is located inside the groove (Fig. 4, B and C). The FAD-binding site is shaped by residues His-142—Arg-151 (the C-terminal end of β5 and the following loop), Thr-183—Thr-185 (the C-terminal end of β7), Asp-247 (the N-terminal end of β11), and Arg-433—Arg-447 (the middle of α14 through the N-terminal end of α15). In addition, residues from the neighboring monomer, Ala-312*—Val-318* (the N-terminal end of α9* and the preceding loop), and Gln-378*—Ser-382* (the C-terminal end of α10* and the following loop) also contribute to the binding of the AMP moiety (asterisks indicate residues
from the neighboring monomer). FAD is stabilized by eight hydrogen bonds with the main chains, seven hydrogen bonds with the side chains, and eight hydrogen bonds with water molecules. Furthermore, the adenine ring of the AMP moiety is stabilized by stacking interactions with side chains of Arg-447 and Tyr-315*.

Despite the conformational changes of loop $\beta5-\beta6$ and loop $\beta8-\beta9$ upon binding of FAD, the groove remains open to the bulk solvent. Accordingly, although the xylene moiety of the isoalloxazine ring is buried in the protein, the re face of the 2,4-pyrimidinedione moiety is accessible to the bulk solvent through ordered water molecules (Fig. 4C). The mouth of the groove is wide enough to allow substrate and molecular oxygen access to the active site after FAD binding. It is worth noting that a water molecule (W1) is bound in the vicinity of the 2,4-pyrimidinedione moiety via hydrogen bonding to Arg-100 and His-142. These residues are likely to select the substrate and define its orientation. Arg-100, Tyr-104, and His-142 are conserved in oxygenases of the TC-FDM family that act on substrates such as 4HPA and phenol, whereas Ser-197 is not conserved in members acting on compounds without carboxyl groups (such as phenol and chlorophenol). The aromatic ring of 4HPA is sandwiched between the side chain of Leu-144 and the main chain carbonyl oxygen of Phe-441 and undergoes further contacts with the side chain of Thr-198, the main chain and side chain of 4HPA (bound in the active site). This distance between the C4a atom of the isoalloxazine ring of FAD and the ortho position of 4HPA is 4.8 Å. This distance is comparable with those of p-hydroxybenzoate hydroxylase (5.3 Å (Protein Data Bank code 1PN0)) (39) and phenol hydroxylase (4.4 Å (Protein Data Bank code 1PBE)) (25).

Interestingly, electron density, which is interpreted as 4HPA, is also observed in a region surrounded by the N-terminal end of $\alpha10$ and the C-terminal end of $\alpha15$ (Fig. 5A). This substrate is stabilized by a polar interaction between the 4-OH group of 4HPA and the side chain of Asp-359 as well as by hydrophobic interactions with the side chains of Leu-452, Val-455, and Tyr-456. Because this substrate is located in the vicinity of the entrance of the active site, it could approach the active site.

The binding mode of FAD of HpaB is similar to that of structurally homologous proteins of 4-hydroxybutyryl-CoA dehydratase from Clostridium aminobutyricum (Protein Data Bank code 1U8V; r.m.s.d. of 2.3 Å for 461 Ca atoms with 24% sequence identity) (37) and pig liver medium chain acyl-CoA dehydrogenase (Protein Data Bank code 3MDD; r.m.s.d. of 2.9 Å for 304 Ca atoms with 13% sequence identity) (38), which contain FAD as a prosthetic group. However, there is a remarkable difference among these proteins. The FAD binding in HpaB causes the conformational change in loop $\beta5-\beta6$. The affinity of HpaB for FAD may be affected by the mobility of loop $\beta5-\beta6$.

The specificity for FADH$_2$ can be explained by the interaction between HpaB and FAD. The side chains of Gln-148 and Arg-151 in loop $\beta5-\beta6$ interact with the phosphate moiety of the AMP portion of FAD. Thus, FMNH$_2$ may not be stabilized by these residues, resulting in the destabilization of loop $\beta5-\beta6$ and the inability to preform the substrate-binding site.

4HPA Binding—The electron density of 4HPA is shown in Fig. 5A. 4HPA (bound in the active site) is located at the re face of the 2,4-pyrimidinedione moiety of the isoalloxazine ring of FAD (Fig. 5B). Upon binding of 4HPA, loop $\beta8-\beta9$ moves significantly and sequesters the active site from the bulk solvent (Fig. 5C). 4HPA is bound to the protein by both hydrophilic and hydrophobic interactions. The carboxyl group of 4HPA is hydrogen-bonded to the side chain of Ser-197 and the main chain nitrogen atom and the side chain of Thr-198. The hydroxyl group of 4HPA forms hydrogen bonds with side chains of Arg-100, Tyr-104, and His-142. These residues are likely to select the substrate and define its orientation. Arg-100, Tyr-104, and His-142 are conserved in oxygenases of the TC-FDM family that act on substrates such as 4HPA and phenol, whereas Ser-197 is not conserved in members acting on compounds without carboxyl groups (such as phenol and chlorophenol). The aromatic ring of 4HPA is sandwiched between the side chain of Leu-144 and the main chain carbonyl oxygen of Phe-441 and undergoes further contacts with the side chain of Thr-198, the main chain and side chain of Phe-442, and the 2,4-pyrimidinedione moiety of FAD. The distance between the C4a atom of the isoalloxazine ring of FAD and the ortho position of 4HPA is 4.8 Å. This distance is comparable with those of p-hydroxybenzoate hydroxylase (5.3 Å (Protein Data Bank code 1PN0)) (39) and phenol hydroxylase (4.4 Å (Protein Data Bank code 1PBE)) (25).

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However, the residues interacting with this substrate are not conserved in the TC-FDM family, and the function of this extra binding site is unclear.

**Possible Oxygen-binding Site**—Monooxygenases use oxygen molecules for substrate hydroxylation and form the C4a-hydroperoxyflavin intermediate. Therefore, the oxygen binding site is expected to be close to the C4a atom of flavin. In the HpaB-FAD+4HPA complex, a water molecule (W2) is bound to a region in the vicinity of the C4a atom of FAD, slightly different from that of W2 (Fig. 6A). These water molecules may indicate the binding site of molecular oxygen as well as the position of the C4a-hydroperoxoy moiety. It should be noted that W2 may mimic a water molecule that is released from C4a-hydroxyflavin.

**Stabilization of the C4a-hydroperoxyflavin Intermediate**—An important question is how the C4a-hydroperoxyflavin intermediate is stabilized until a substrate enters the binding site. As described above, Arg-100 is located in the vicinity of the oxygen-binding site. In the HpaB-FAD complex, the Ne atom of Arg-100 is located 4.8 Å from the C4a atom of FAD, and the Nη atom of Arg-100 is located 2.6 Å from the water molecule (W1). If the C4a-hydroperoxyflavin intermediate is formed, the Ne atom of Arg-100 can form a hydrogen bond with the putative peroxy moiety of the C4a-hydroperoxyflavin intermediate (with a minor conformational change in the side chain). This indicates that Arg-100 may contribute to the formation and/or stabilization of the C4a-hydroperoxyflavin intermediate. Arg-100 is conserved in the *E. coli*-type oxygenases of the TC-FDM family.

It is worth noting that the N5 atom of the isoalloxazine ring forms a hydrogen bond with the side chain of Thr-185. This hydrogen bond extends to a salt bridge network formed by Asp-247, Arg-225, Arg-433, and Glu-244. Bach and Su (40) have suggested that, on the basis of *ab initio* molecular orbital calculations, a hydrogen bond to the N5 hydrogen of the C4a-hydroperoxyflavin intermediate could afford a stabilized transition structure for oxygen transfer, providing a novel explanation for the unusual monooxygenase reactivity of flavins. Thr-185 as well as other residues in the salt bridge network appear to be mostly conserved in the *E. coli*-type oxygenases (Fig. 2).

These contrast to the case of single-component monooxygenases. In these enzymes substrates are bound to the active site before the formation of the C4a-hydroperoxyflavin intermediate, so substrate hydroxylation immediately occurs upon formation of the C4a-hydroperoxyflavin intermediate (21–25, 41, 42).
Crystal Structure of HpaB from Thermus thermophilus

42. This indicates that, in single-component systems, the stabilization of the C4a-hydroperoxoflavin intermediate requires the bound substrate (23). In fact, structures of these enzymes do not reveal residues that should be involved in the stabilization of the C4a-hydroperoxoflavin intermediate. Instead, it appears that single-component enzymes and tryptophan 7-halogenase (43, 44) as well form a cavity in proximity to flavin to accommodate molecular oxygen as well as the hydroperoxy moiety of the C4a-hydroperoxoflavin intermediate (45).

Implications for the Catalytic Mechanism—Our proposed catalytic mechanism is as follows (Fig. 7). (i) A reduced FAD, which is supplied from HpaC, binds to HpaB and is stabilized by loop β5–β6. The substrate-binding site (Ser-197 and Thr-198) is preformed. (ii) The reduced FAD accepts an oxygen molecule at the C4a position of the isoalloxazine ring and forms the C4a-hydroperoxoflavin intermediate, which is facilitated and stabilized by Arg-100. (iii) 4HPA binds to the enzymes-C4a-hydroperoxoflavin complex and is stabilized by residues His-142, Tyr-104, Arg-100, Ser-197, and Thr-198. 4HPA is activated by His-142 via deprotonation of the 4-hydroxyl group, and a hydroxyl group of the C4a-hydroperoxoflavin intermediate is introduced into the ortho position of 4HPA by electrophilic attack, yielding a dienone form of the product. (iv and v) Rearomatization of the dieneon yield DHPA and C4a-hydroxyflavin. (vi) DHPA dissociates from HpaB. (vii) A water molecule is introduced into the 4HPA and phenol as a substrate. In addition, the carboxyl group of 4HPA and phenol as a substrate. In addition, the carboxyl group of 4HPA forms a salt bridge with Arg-263 in C2, whereas that of 4HPA forms a hydrogen bond with Ser-197 and Thr-198 in HpaB.

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