Crystal Structure of 3-Hydroxybenzoate Hydroxylase from Comamonas testosteroni Has a Large Tunnel for Substrate and Oxygen Access to the Active Site

Takeshi Hiromoto1, Shinsuke Fujiwara2, Keiichi Hosokawa3,4 and Hiroshi Yamaguchi1⁎

1Department of Chemistry Nanobiotechnology Research Center, School of Science and Technology, Kwansei Gakuin University, 2–1 Gakuen, Sanda Hyogo 669–1337, Japan
2Department of Bioscience Nanobiotechnology Research Center, School of Science and Technology, Kwansei Gakuin University, 2–1 Gakuen, Sanda Hyogo 669–1337, Japan
3Institute of Bio-Microbiology 1–5–12 Akasaka, Minato Tokyo 107–0052, Japan
4Proteomics Research Laboratory, 1–16–1 Amakubo Tsukuba, Ibaraki 300–0005 Japan

The 3-hydroxybenzoate hydroxylase (MHBH) from Comamonas testosteroni KH122-3s is a single-component flavoprotein monooxygenase, a member of the glutathione reductase (GR) family. It catalyzes the conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate with concomitant requirements for equimolar amounts of NADPH and molecular oxygen. The production of dihydroxy-benzenoid derivative by hydroxylation is the first step in the aerobic degradation of various phenolic compounds in soil microorganisms. To establish the structural basis for substrate recognition, the crystal structure of MHBH in complex with its substrate was determined at 1.8 Å resolution. The enzyme is shown to form a physiologically active homodimer with crystallographic 2-fold symmetry, in which each subunit consists of the first two domains comprising an active site and the C-terminal domain involved in oligomerization. The protein fold of the catalytic domains and the active-site architecture, including the FAD and substrate-binding sites, are similar to those of 4-hydroxybenzoate hydroxylase (PHBH) and phenol hydroxylase (PHHY), which are members of the GR family, providing evidence that the flavoprotein aromatic hydroxylases share similar catalytic actions for hydroxylation of the respective substrates. Structural comparison of MHBH with the homologous enzymes suggested that a large tunnel connecting the substrate-binding pocket to the protein surface serves for substrate transport in this enzyme. The internal space of the large tunnel is distinctly divided into hydrophilic and hydrophobic regions. The characteristically stratified environment in the tunnel interior and the size of the entrance would allow the enzyme to select its substrate by amphiphilic nature and molecular size. In addition, the structure of the Xe-derivative at 2.5 Å resolution led to the identification of a putative oxygen-binding site adjacent to the substrate-binding pocket. The hydrophobic nature of the xenon-binding site extends to the solvent through the tunnel, suggesting that the tunnel could be involved in oxygen transport.

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*Corresponding author

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Introduction

Various kinds of aromatic compounds derived from lignin, a structural component of plants, serve as growth substrates for numerous aerobic soil bacteria.1 Flavin-containing aromatic hydroxylase is a monoxygenase that functions in the first step of the aerobic catabolic pathways for phenolic compounds.2,3 It catalyzes hydroxylation of the aromatic
substrate by the addition of one atom of molecular oxygen with consumption of an equimolar amount of NAD(P)H as an electron donor. The resulting dihydroxybenzenoid compounds are subjected to ring-cleavage pathways that lead to tricarboxylic acid cycle intermediates. The enzymes involved in the metabolic pathways play an indispensable role in the mineralization of organic compounds by soil microorganisms, the natural scavengers in the environment.

*Comamonas testosteroni* strain KH122-3s is an aerobic soil bacterium that utilizes 3 or 4-hydroxybenzoate as a sole carbon and energy source, whereas it does not utilize salicylate. In this strain, 3 and 4-hydroxybenzoate are catalyzed to 3,4-dihydroxybenzoate by 3-hydroxybenzoate hydroxylase (MHBH, EC 1.14.13.23) and 4-hydroxybenzoate hydroxylase (PHBH, EC 1.14.13.2), respectively. The dihydroxyalted compound serves as a substrate for a ring-fission dioxygenase that cleaves the aromatic ring between a dihydroxylated carbon and an adjacent non-hydroxylated carbon (meta-cleavage). The hydroxylation of 3-hydroxybenzoate is an important criterion for classification of *C. testosteroni* and *Comamonas acidovorans*. The former hydroxylates at the C4 (ortho) carbon to form 3,4-dihydroxybenzoate, while the latter hydroxylates at the C6 (para) carbon to form 2,5-dihydroxybenzoate.

Previously, we reported some properties of MHBH. The enzyme is an FAD-dependent monooxygenase that catalyzes the conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate with a specific requirement for NADPH (Figure 1(a)). Its substrate specificity is generally high under the experimental conditions, whereas MHBH shows activities for 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate that are 31.7 and 17.0%, respectively, of the activity for 3-hydroxybenzoate (100%). In the present work, we cloned the entire MHBH coding region from the *Comamonas* chromosome and determined the deduced amino acid sequence. The sequence of MHBH defines it as a member of the flavoprotein aromatic hydroxylase class in the glutathione reductase (GR) structural family. Among the enzymes, three sequence motifs are highly conserved for binding of the cofactor FAD.

In the GR family, 4-hydroxybenzoate hydroxylase (PHBH, E.C. 1.14.13.2) from *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa*, and phenol hydroxylase (PHHY, E.C. 1.14.13.7) from *Trichosporon cutaneum* are well characterized as single-component flavoprotein aromatic hydroxylases. The catalytic mechanisms have been investigated thoroughly by many structural and kinetic studies. The enzymes share a common catalytic mechanism consisting of two reaction steps; (i) the reduction of enzyme-bound FAD with NADPH in the presence of the aromatic substrate; and (ii) the oxidation of reduced FAD with molecular oxygen, generating a highly reactive intermediate, flavin C4a-hydroperoxide, and then its hydroxyl group is introduced into the substrate by electrophilic aromatic substitution. The two-step reaction proceeds with conformational changes of FAD. The rearrangement of the isoalloxazine moiety of FAD has been shown to be a key step in catalysis as well as in substrate transport in both enzymes. On the basis of structural studies of PHBH, it has been proposed that the substrate is transported from the re-side of the isoalloxazine ring to the substrate-binding pocket in the protein interior through a solvent channel, as in PHHY. The solvent channel is opened when the isoalloxazine ring adopts the “open” position and is exposed to the solvent region. After binding with the substrate, the flavin moves to the “in” position located closer to the substrate-binding pocket, in which the hydroxyl group of the substrate is deprotonated through an internal hydrogen-bond network connected to the external solvent. This substrate deprotonation is an essential step for catalysis, because the resulting phenoxide ion markedly increases the nucleophilicity of the ortho carbon in the phenolic substrate to be hydroxylated. Furthermore, the negative charge generated on the aromatic ring stimulates a conformational change allowing the flavin to rearrange to the “out” position, where the flavin is more exposed to the solvent region and has the possibility of forming a complex with NADPH. Because the deprotonation of substrate plays an important role in control of the progression of the catalytic process, it has been thought that it takes part in the final step of substrate recognition. Finally, the reduced FAD takes the “in” position and reacts with oxygen in the protein interior to form the flavin C4a-hydroperoxide, which subsequently attacks at the ortho carbon atom of the phenolic substrate. Recent mechanistic investigations of PHHY, however, indicated that the hydrogen bond network found in this enzyme is not involved in substrate deprotonation but provides an environment to support the oxidation of reduced FAD. Although the enzymes, in general, have similar reaction intermediates, the actual mechanism of substrate hydroxylation may vary from one member to another within the flavoprotein aromatic hydroxylase class of the GR family.

The mechanistic similarity is reflected in a high degree of structural similarity between PHBH and PHHY. The protein fold of the catalytic domains and the ligand locations are well conserved between them, whereas they can recognize the respective genuine substrates by shape and size, and catalyze the site-specific hydroxylation. To gain further insight into the structural requirements for specific recognition of the aromatic substrate, we determined the crystal structure of MHBH in the Michaelis complex form. Moreover, analysis of the Xe-derivative structure identified a highly hydrophobic pocket adjacent to the substrate-binding site. These findings would account for how each substrate and molecular oxygen are transported to the active site, and how they are recognized by this enzyme.
Results

Primary structure

In an attempt to obtain the nucleotide sequence of the MHBH coding region, Southern analysis of the C. testosteroni chromosome was performed with a probe that was prepared by PCR using primers based on the N-terminal and internal amino acid sequences (see Materials and Methods). An approximately 4.0 kb PstI fragment was detected and subsequently cloned. Sequence analysis of the isolated fragment identified one open reading frame (ORF) of 1917 nucleotides downstream of a putative ribosomal binding site, ATG, which is located at six bases downstream of the initiation codon, ATG, which is located at six bases downstream of a putative ribosomal binding sequence (GAGG).8 We named this gene mobA. It was predicted to encode a protein with 639 amino acid residues with a calculated molecular mass of 70 kDa.

The deduced amino acid sequence shows the highest similarity (35% identity in 664 amino acids, Swiss-Prot accession no. P15245) to that of PHHY from the basidiomycete yeast Trichosporon cutaneum11,16 in the SWISS-PROT protein sequence database as detected with the program BLASTP†. Other monooxygenases, pentachlorophenol hydroxylase from Escherichia coli (25% identity in 386 amino acids, P42535),32 tetracenomycin polypeptide synthase hydroxylase from Streptomyces glaucescens (24% identity in 406 amino acids, P39888),33 2,4-dichlorophenol hydroxylase from Cupriavidus necator (27% identity in 398 amino acids, P27138)34 and PHHY from Pseudomonas sp. (26% identity in 200 amino acids, P31020),35 were also detected but indicated relatively low homology to MHBH. In particular, PHBH from Pseudomonas fluorescens (P39888)33 shows a lower sequence homology (23% identity in 219 amino acids, P00438)13 and lacks approximately 120 residues that correspond to the C-terminal part of MHBH. On the other hand, alignment of MHBH with PHHY and PHBH shows three consensus sequences (residues 35–48, 201–214 and 342–372) that are highly conserved for binding of the FAD cofactor in the flavoprotein aromatic hydroxylase class of the GR family (Figure 1(b)),11,12 indicating that MHBH is a typical monooxygenase belonging to this class. The first FAD-binding motif located in the N-terminal region is a part of the well-known Rossmann fold, which is broadly shared by all GR family members. Other members of the GR family have another sequence motif for a Rossmann-type NADPH-binding fold, but the motif is not conserved in the flavoprotein aromatic hydroxylase class of enzymes.1,35 Instead, it has been proposed that the second FAD-binding sequence motif is involved in binding of both FAD and NADPH in this class.

Overall architecture

The crystal structure for MHBH in complex with its substrate at 1.8 Å resolution and the other for Xe-derivative at 2.5 Å resolution were determined. These crystals belong to the trigonal space group P321 with one molecule in the asymmetric unit. Initial phases for a substrate–complex crystal were calculated to 3.0 Å resolution by the multiple isomorphous replacement with anomalous scattering (MIRAS) technique. The initial model was built manually and then refined against the high-resolution data of substrate complex and the Xe-derivative data, respectively. Details of the crystallographic data statistics are presented in Table 1. The Xe-derivative structure was superimposed on the substrate complex structure with a root-mean-square deviation (rmsd) of only 0.2 Å for 608 Ca atoms by using the Dali server,37 indicating that the overall structures are essentially identical except for xenon binding. The substrate complex structure is described here, unless noted otherwise.

The MHBH structure shows the physiological form of a homodimer with overall dimensions of ~100 Å×110 Å×70 Å, in which each subunit is related by a crystallographic 2-fold axis (Figure 2(a)). The subunit is divided into three α/β domains; the catalytic domains I and II, and the C-terminal domain III (Figure 2(b)). On the basis of the CATH structure classification,38 domain I has a three-layer (ββα) sandwich, in which a three-stranded antiparallel β-sheet (β8, β9 and β10) and a three-stranded parallel β-sheet (β1, β2 and β10) including the Rossmann fold form one β-sandwich surrounded by a large helical component (H1, H2, H3, H4, H9, H10, H11 and H12). Domain II is a two-layer sandwich with a seven-stranded β-sheet (β5, β6, β12, β13, β14, β15 and β16) and a helix, H7. Each one molecule of 3-hydroxybenzoate and FAD is located in a cleft formed between domains I and II. Domain III is composed of the typical thioredoxin fold39 which has a central four-stranded mixed β-sheet (β24, β25, β29 and β30) and five flanking helices (H13, H15, H16, H20 and H21), plus additional elements that consist of four short 30ω-helices (H14, H17, H18 and H19) and six β-strands (β21, β22, β26, β27 and β28). The additional elements are arranged on one side of the thioredoxin fold and form part of the dimer interface. In the interface, the N-terminal loop region of domain I (residues 1–13) and the additional elements of domain III (H17, H18, H19, β22, β23, β27 and their neighboring loops) make contact with domain I (H3, H4, β4 and a loop region between β2 and β3) and domain II (β5, β6, β12, β15, β16 and their neighboring loops) of another subunit, with a solvent-accessible surface of 2583 Å² buried upon dimerization. A large part of the interface is built of direct or water-mediated hydrogen bonds, with hydrophobic interactions in the remaining area. One intermolecular salt-bridge exists between the side-chains of Asp128 and Lys889.

FAD-binding site

One FAD molecule is bound in an elongated cleft on the protein surface between domains I and II (Figures 2(b) and 3(a)). A large part of FAD is exposed to the solvent region. The FAD-binding site is formed predominantly by amino acid residues from domain I, which includes the Rossmann fold. The protein surface around the cleft is rich in positively charged residues, which would be a favorable property in binding of negatively charged NADPH during catalysis. The isoalloxazine ring moiety is located at one end of a large tunnel that runs through the protein interior in the direction opposite to the FAD-binding site (Figure 3(b)). In the cleft, the N3 and O2 atoms of the isoalloxazine ring form hydrogen bonds with the side-chain of Tyr271 and a water molecule, respectively (Figure 4). The aromatic ring of Tyr317 stacks in parallel with the re-side of the isoalloxazine ring at a distance of 3.6 Å. Such a π–π stacking interaction between an aromatic residue and the isoalloxazine ring is found in other flavin-containing enzymes, in which the aromatic side-chain plays important roles in FAD binding and catalytic action. The hydrophobic portion of Gln73 forms a contact with the si-side of the isoalloxazine ring, which fixes its orientation. It should be noted that the atoms of the isoalloxazine ring and the ribityl chain have higher B-factors than the rest of the structure, and the electron densities of these moieties are not well defined. These facts suggest that FAD has considerable flexibility; therefore, the conformational changes in FAD that occur during the catalysis would be similar to those in PHHY and PHBH.

Substrate-binding site and a large tunnel in the protein interior

The electron density of the substrate, 3-hydroxybenzoate, was clearly observed in a pocket adjacent to the FAD-binding site (Figure 5). The substrate-binding pocket is composed of amino acid residues from domains I and II. The side-chains of Asp75 and Tyr227 form a polar edge on the lip of the pocket, and the positively charged side-chains of His135 and Lys247 form the rest of the edge. In the pocket, the 3-hydroxyl group of the substrate makes hydrogen bonds with the side-chains of Asp75 and Tyr227, and the oxygen atoms of its carboxyl group form salt-bridges with the side-chains of His135 and Lys247. In addition, Asp75 establishes a hydrogen-bond network leading to the solvent region with the side-chains of Arg262 and Glu263 (Figure 7(a)). The benzene ring of the substrate faces a hydrophobic surface formed by the side-chains of Leu258 and Ile260 at the bottom of the pocket. The shape of the binding pocket closely resembles that of the substrate molecule.

The substrate-binding pocket lies at the bottom of the large tunnel in the protein interior (Figure 3(b)). The tunnel has two entrances, E1 and E2, open to the solvent region. The entrances are separated on the protein surface by hydrophobic residues Phe106, Val112 and Phe435 but join in the protein interior, forming one large space. Entrance E1 has an elongated shape with dimensions of \( \sim 8 \times 15 \) Å, and entrance E2 has a diameter of approximately 8 Å. The pathway from E1 to the substrate-binding pocket is composed of hydrophobic side-chains and hydrophobic portions of some polar residues, where any solvent molecules are kept away. In contrast, the pathway through the entrance E2 is rich in polar residues and is filled with many water molecules. No such large tunnel exists in either PHHY or PHBH. These findings suggest that MHBH adopts a unique pathway for substrate transport that differs from that of the homologous enzymes. The external surface around both entrances is formed mainly by polar residues. Interestingly, near E1, Lys108 and His120 are arranged with a geometry that is similar to that of Lys247 and His135 in the substrate-binding pocket (Figure 3(a)). These residues may initiate the first binding of substrate in order to transport it to the active site, as proposed for PHBH.

Structural comparison of MHBH with the homologous proteins

Structures homologous to MHBH were searched for in the Protein Data Bank by means of the Dali server. The overall architecture is closest to that of PHHY (PDB entry 1PN0) with an rmsd of 1.9 Å for 553 Cα atoms, as predicted from the sequence homology (Figure 6(a)). PHHY has a three-domain structure with a topology nearly identical with that of MHBH. Its C-terminal domain also contains the thioredoxin fold and contributes to the dimer formation. Superimposition of these structures, however, shows clearly that MHBH lacks the sequence corresponding to residues 170–210 in PHHY. This inserted region of PHHY is located near the isoalloxazine ring of FAD and is mobile, in accordance with conformational changes of FAD. Hence, it has been proposed that the flexible part serves as a lid for the solvent channel that connects the surrounding solvent region to the substrate-binding pocket of the protein interior across the re-side of the isoalloxazine ring. Another structural difference is that helices H11 and H12 of MHBH are placed more laterally than the equivalent helices of PHHY, forming the tunnel in the protein interior. The second-closest relative is PHBH (PDB entry 1PBE) with an rmsd of 2.8 Å for 355 Cα atoms. It has only two catalytic domains due to deletion of the C-terminal region that corresponds to domain III conserved between MHBH and PHHY. Therefore, the intermolecular interactions for dimerization in PHBH differ substantially from those in MHBH and PHHY. However, the folding topology and relative orientations of catalytic domains I and II of MHBH are fundamentally similar to those of PHHY and PHBH. The FAD and substrate-binding sites are located in equivalent positions between the two catalytic domains for all three structures. The structural similarities may support the proposal that members of the flavoprotein aromatic hydroxylase superfamily would have similar fold and functional properties.

Figure 1 (legend on opposite page)
Table 1. Data collection, phasing and refinement statistics

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* Values in parentheses are for the highest-resolution shells.

** R free = Σhkl |Fhkl| − Σhkl |Ihkl| / Σhkl |Fhkl|, where |Ihkl| is the mean measured diffraction intensity and |Fhkl| is the mean intensity for the Miller index (hkl).

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class share a similar catalytic mechanism for substrate hydroxylation.21

The binding mode with FAD is very similar to that of PHHY (Figure 4). In PHHY17 and PHBH,15,16 the catalytic process comprised of reductive and oxidative reaction steps is driven by conformational changes of FAD between the in position (buried in the protein interior) and the out position (exposed to the external solvent region). The conformation of FAD in the crystal structure of MHBH corresponds to the out position. A notable structural feature of MHBH is that there is no solvent-accessible channel around the isoalloxazine ring. In both PHHY and PHBH, the isoalloxazine ring takes the out or open position to form a solvent channel for substrate access as described above. However, in the MHBH structure, the bulky side-chain of Tyr317 lies on the re-side of the isoalloxazine ring (Figure 3(b)), and the substrate would not be directly accessible to the substrate-binding pocket through the re-side of the isoalloxazine ring. No amino acid residue is localized in the corresponding position in the PHHY and PHBH structures.

The geometrical arrangement of residues forming the substrate-binding pocket is the same as that in PHHY. In MHBH, Tyr271 and Asp75 occupy positions corresponding to those of Tyr289 and Asp54, respectively, which interact with the hydroxyl group of phenol in the substrate complex structure of PHHY (Figure 7(b)). Asp54 residue also forms a hydrogen bond network that is connected to the solvent region through the side-chain of Arg281. It

Figure 1. (a) The hydroxylation reaction catalyzed by MHBH. (b) Alignment of the amino acid sequences from: top, MHBH; middle, PHHY (PDB entry 1PN0)18 and bottom, PHBH (PDB entry 1PBE).19 The sequence alignment was carried out with CLUSTAL W.69 The secondary structure elements of MHBH, assigned by PROMOTIF,70 are colored according to the respective domains (domain I, green; domain II, yellow; domain III, blue). Identical residues in the alignment are shaded gray. Residues involved in substrate binding are indicated by red letters, and those involved in FAD binding are shaded pink. Three boxes colored violet denote the conserved sequence motifs for FAD binding in the flavoprotein aromatic hydroxylase class of the GR family.11 Green boxes in the MHBH sequence indicate amino acid residues that could be involved in oxygen binding, as found in this study. The corresponding residues in the structures of PHHY and PHBH are indicated. Residues forming the proton-transfer pathway in PHBH are shaded cyan.20
was anticipated that the hydrogen bond network would be involved in proton transfer for substrate deprotonation as found in PHBH.\textsuperscript{25,26} However, subsequent mutation studies of PPHY indicated that the catalytic process is not mediated in the phenolate state of the substrate.\textsuperscript{20} Instead, the

**Figure 2.** Crystal structure of MHBH in complex with its substrate. (a) The overall structure of the MHBH dimer is shown in two orthogonal orientations: left, top view of the dimer interface; and right, side view rotated 90°. Domains I, II and III are colored green, yellow and blue, respectively. The loops that were not built into the structure due to the absence of electron density are shown as dotted lines. The 3-hydroxybenzoate and FAD molecules are represented as stick models and colored pink and yellow, respectively. (b) A ribbon diagram of the monomer viewed from the right side of the dimer shown in the left view of (a).
residues are considered to be involved in the effective oxidation of reduced FAD by providing a suitable environment for the reaction. In contrast, the residues that form hydrogen bonds with the substrate as well as the orientations of FAD and substrate molecules differ substantially from those of PHBH (Figure 7(c)).

Interestingly, MHBH and PHHY contain the typical thioredoxin fold in the C-terminal domain III.17 This fold is conserved in all members of the peroxiredoxin family of peroxidases.39,44 The enzymes share one catalytic cysteine residue in the N-terminal regions and are able to reduce peroxide with thioredoxin or other thiol-containing reagents as electron donors. Domain III of MHBH has a cysteine residue, Cys521, corresponding to Cys47 in the active site of a human peroxiredoxin, hORF6 (PDB entry 1PRX; with an rmsd of 3.0 Å for 120 Cα atoms),45 which reduce hydrogen peroxide in the presence of dithiothreitol (Figure 6(b)). During the

Figure 3. Molecular surface representation of the MHBH monomer. (a) The surface viewed from: left, the side of the FAD-binding site; and right, from the direction rotated approximately 180° around the vertical axis. The protein surfaces are shown by electrostatic potential isocontours from the potential of +8 kT e⁻¹ (blue) to −8 kT e⁻¹ (red).65 The FAD molecule is shown as a stick model, as in Figure 2(a). The region rich in positive charged residues around the FAD-binding site is circled with a dotted line. The region might serve as a binding site for NADPH. E1 and E2 indicate the entrances of a tunnel leading to the active site. Only amino acid residues discussed in the text are indicated. (b) Cross-section view of the active-site surface showing a large tunnel in the protein interior. The entrances are circled in green. The pathway from E1 to the substrate-binding pocket is composed of hydrophobic side-chains and hydrophobic portions of some polar residues, where any solvent molecules are kept away. In contrast, the pathway through the E2 entrance is rich in polar residues and is filled with many water molecules.
catalytic process with flavoprotein aromatic hydroxylases, cytotoxic hydrogen peroxide is released as a by-product through hydrolysis of the flavin C4a-hydroperoxide intermediate. It is speculated that MHBH has acquired the additional peroxidase domain during its evolution to protect cells from oxidative attack by hydrogen peroxide, but the activity has not been measured. Furthermore, no corresponding cysteine residue is conserved in PHHY.

Xe derivative structure

Flavoprotein aromatic hydroxylases require molecular oxygen for substrate hydroxylation. It was proposed that the binding site for oxygen in the protein interior is required to form the C4a-hydroperoxide intermediate in the enzymes; however, until recently there has been only limited experimental evidence to support the hypothesis. We have therefore sought to detect experimentally whether
possible oxygen-binding sites or oxygen transport pathways exist near the active site. The xenon atom is a non-polar gas like molecular oxygen and has a large atomic number. Under high-pressure conditions, xenon could occupy potential oxygen-binding sites (e.g. hydrophobic regions) in protein interiors, due to its hydrophobic nature. X-ray crystallographic analysis of the Xe-derivatives therefore is a convenient way to identify the binding sites.

Three strong electron density peaks were found from an $F_{\text{o(xenon)}} - F_{\text{o(substrate)}}$ electron density map calculated with the phase information of the substrate complex structure (Figure 8(a)). These peaks were confirmed in an anomalous difference electron density map generated by using the anomalous X-ray scattering of xenon. In the substrate complex structure, no electron density corresponding to any solvent molecules was

**Figure 6.** (a) Superposition of the Cα traces of MHBH and PHHY (PDB entry 1PN0). MHBH and PHHY are colored blue and gray, respectively. Each molecule of 3-hydroxybenzoate and FAD in the MHBH structure is colored purple and yellow, respectively. For comparison with MHBH, the insertion segment of PHHY (residues 170–210) is shown in red. (b) The ribbon diagrams of: left, domain III (residues 453–639) of MHBH; and right, the human peroxiredoxin, hORF6 (PDB entry 1PRX). Conserved cysteine residues, Cys521 in domain III and Cys47 located at the active site of hORF6, are shown as stick models.
observed at either of the Xe-binding sites. Furthermore, there is no electron density of the substrate molecule in its binding pocket of the Xe-derivative structure; instead, the pocket is filled with some water molecules. This replacement may be due to collision between the substrate and xenon atoms.

One xenon atom, with the highest occupancy of 0.35, binds to a hydrophobic pocket formed by Pro356 and Lys357 from domain I, and Trp230, Leu258 and Tyr271 from domain II (Figure 8(b)). The hydrophobic pocket is located near the substrate-binding site. Comparison with the substrate complex structure indicated that the C4 atom of the substrate is close to the xenon atom, at a distance of 5.1 Å. The atomic positions of the Xe-derivative structure are not so accurate, with a Cruickshank’s DPI value of 0.46 Å due to the resolution limit of 2.5 Å (Table 1). However, it is a fact that the distance between the Xe-binding site and the C4 atom to be hydroxylated is short enough. Therefore, it would be possible that the hydrophobic pocket serves as a specific binding site for molecular oxygen. In addition, the residues forming the hydrophobic pocket tend to be conserved in both the PHHY and PHBH structures (Figure 1(b)). Another Xe-binding site, with an occupancy of

Figure 7. Comparison of the active site in (a) MHBH with those in (b) PHHY and (c) PHBH. The respective substrates and FAD molecules are shown as stick models, as in Figure 2(a). The molecular surfaces of each substrate-binding pocket are shown in pale yellow. The spatial shapes of the pockets are nearly identical with the molecular structures of the respective substrates, which would prevent the enzymes from binding incorrect compounds at their pockets. Amino acid residues that make contact with the substrate are colored and labeled. Residues predicted to be involved in substrate deprotonation are shown in cyan. The proline residue (pale yellow) that is conserved near the substrate-binding pocket in the enzymes is expected to stabilize the flavin C4a-hydroperoxide intermediate.
0.30, is located in a small cavity in domain III (Figure 8(a)). This site consists also of hydrophobic residues Leu501, Leu524, Pro531, Phe546, Leu629 and Phe633. Cys521, conserved in the peroxiredoxin family as a catalytic residue, exists in the same cavity at a distance of 10.4 Å from the xenon atom. This may imply that the hydrophobic pocket serves as a binding site for hydrogen peroxide or reducing agent, if domain III possesses the peroxidase activity. The remaining third xenon atom is located on the surface of domain III, where a hydrophobic pocket is formed with another dimer related by a crystallographic $2_1$ screw axis.

Superimposition of the active site of the Xe-derivative structure on that of the substrate complex shows that the positions and conformations of the surrounding residues are nearly identical, and only Asp75 indicates a subtle conformational difference. Due to the lack of the substrate molecule in the Xe-derivative, the side-chain of Asp75 rotates slightly, resulting in distortion of the hydrogen bond between Asp75 and Arg262. The formation of the hydrogen bond network leading to the solvent in the substrate complex structure may allow the enzyme to recognize the hydroxyl group of the substrate and control the progress of its catalytic reaction.

**Discussion**

**Substrate transport pathway**

The structural features of MHBH, the characteristic two-domain folding topology and the relative locations of the FAD and substrate-binding sites, are fundamentally similar to those of PHHY as well as PHBH. However, the large tunnel that connects the active site and external solvent was found between the catalytic domains only in MHBH, which would imply that the substrate transport pathway should differ from that of the homologous enzymes.

According to structural studies of PHHY and PHBH, the conformational change of FAD to the out or open position induces formation of a solvent-accessible channel across the $re$-side of the isoalloxazine ring. In PHBH, the substrate transport is initiated by binding to a low-affinity site located near the channel entrance, and then the substrate is guided into the high-affinity substrate-binding pocket through the channel. PHHY has a flexible element located in the vicinity of the FAD-binding site, and it has been proposed that the flexible part serves as a lid for the solvent channel (Figure 6(a)).

Instead of the solvent channel pathway across the $re$-side of the isoalloxazine ring, MHBH would be able to use a large tunnel in the protein interior for substrate transport (Figure 3(b)). This tunnel connects the substrate-binding pocket to the protein surface with two entrances. Entrance E1 opens more widely to the solvent region than entrance E2. Basic residues, Lys108 and His120, around E1 are arranged in positions similar to those of His135 and Lys247 in the substrate-binding pocket, suggesting that the positively charged residues provide an initial binding site for substrate, as proposed for
PHBH (Figure 3(a)). In addition, the internal space of the tunnel is divided distinctly into hydrophilic and hydrophobic regions. The genuine substrate, 3-hydroxybenzoate, has hydrophilic substituents with its hydrophobic benzene ring. If the substrate is introduced from the wider opening E1, its polar and hydrophobic portions would interact with the hydrophilic and hydrophobic regions of the internal space, respectively, and the substrate is guided smoothly into the substrate-binding pocket with a pertinent orientation. At the bottom of the tunnel, the negatively charged carboxyl group of the substrate would interact preferentially with His135 and Lys247, which form a part of the substrate-binding pocket. The stratified environment of the tunnel interior would therefore play an important role for determining the substrate orientation. After hydroxylation, 3,4-dihydroxybenzoate, a more hydrophilic compound than the substrate, might be released into the external solvent through the hydrophilic region leading to E2. According to this transport model, the large tunnel in MHBH would facilitate the selection and uptake of the substrate by its molecular size and amphiphilic nature, allowing smooth product release.

**Structural basis for substrate recognition**

Comparison of the molecular machineries for substrate transport and binding in MHBH with those in PHHY and PHBH allowed us to assign the structural determinants for specific substrate recognition. As suggested above, the substrate molecule might encounter the first selection at an initial binding site on the protein surface, and then its transport would secondly be restricted by passage through the tunnel. Even if other candidates for the substrate are carried into the protein interior, all except for the genuine substrate would not be accepted in its binding pocket due to mismatch with the steric arrangement of the amino acid residues that form the pocket (Figure 7(a)). Thus, MHBH discriminates the isomers 4-hydroxybenzoate and salicylate distinctly from 3-hydroxybenzoate.

In the previous biochemical characterization, MHBH showed high substrate specificity under the experimental conditions, whereas relative activities for 2,3, 2,5 and 3,5-dihydroxybenzoate were 31.7%, 17.0% and 5.0%, respectively, of that for 3-hydroxybenzoate (100%). It appears that the decrease of the catalytic activity, suggesting the subtle distortion of the molecular orientation in the pocket results in a decrease of the hydroxylation efficiency.

In conclusion, the substrate-recognition process in MHBH consists of three steps; (i) the initial binding on the protein surface; (ii) the transport to the active site through the amphiphilic tunnel; and (iii) the accurate binding to the substrate-binding pocket, which enable MHBH to react with its substrate with highest efficiency and specificity.

**Putative oxygen binding site**

On the basis of both model studies in PHBH and the requirement for spin inversion, the reaction of reduced FAD with molecular oxygen is not favorable thermodynamically, and therefore it does not take place until oxygen is in direct contact with reduced FAD in the pocket provided by the protein. Furthermore, many water molecules were present around the active site in the substrate complex structure of MHBH (Figure 3(b)). The exclusion of water molecules from the active site would be required to prevent the intermediate from decomposition to oxidized FAD and hydrogen peroxide.

The Xe-derivative structure of MHBH revealed the presence of a high-affinity Xe-binding site in the vicinity of the substrate-binding pocket (Figure 8(b)). Comparison of the Xe-derivative structure with the substrate complex structure shows that the Xe-binding site is close enough to the C4 atom of the substrate at a distance of about 5 Å. This raises the possibility that the hydrophobic pocket is a specific binding site for molecular oxygen. According to structural and computational studies of PHHY and PHBH, it has been proposed that the distal oxygen atom of the flavin C4a-hydroperoxide intermediate forms a hydrogen bond with the carbonyl oxygen atom of a proline residue near the substrate-binding pocket to stabilize the hydroperoxide (Figure 7(b) and (c)). In the Xe-derivative structure, the carbonyl oxygen atom of the corresponding Pro356 residue is 4.1 Å distant from the xenon atom (Figure 7(a)). The C4a atom of the isoalloxazine ring is approximately 9 Å distant from the xenon atom; however, the conformational change of the isoalloxazine moiety to the in position would enable it to activate an oxygen molecule at the putative binding site. In addition, the existence of the hydrophobic region within the large tunnel could facilitate recruitment of molecular oxygen due to its hydrophobic nature. Thus, it appears reasonable that molecular oxygen is supplied from external solvent to the putative oxygen-binding site by passage through the hydrophobic region of the tunnel, then storage at its binding site in order to carry out subsequent reactions effectively.

**Structural variations in the catalytic action**

Members of the flavoprotein aromatic hydroxylase class show similar catalytic actions for substrate hydroxylation due to their structural similarities. In the MHBH structure, FAD occupies predominantly the out position in the presence of the substrate (Figure 4). High B-factor values of the isoalloxazine moiety suggest that the catalytic process is accompanied by conformational changes of FAD, as found in both PHHY and PHBH. The
isoalloxazine ring of FAD also occupies the out position in the Xe-derivative structure, although the substrate is absent from the binding pocket. This observation suggests that the conformational change to the in position, which is required to react with the substrate, should take place by NADPH binding or FAD reduction by NADPH rather than substrate binding.\textsuperscript{21,28,29} Indeed, co-crystallization of MHBH with NADPH in the presence of the substrate was failed. Substrate complex crystals broke down when they were soaked in crystallization buffer containing NADPH.

The presence of a tyrosine residue, Tyr317, stacking to the isoalloxazine ring is one of the significant differences among the active sites of the three structures (Figure 3(b)). Since the electron density of Tyr317 is low, it might move with the conformational change of FAD or independently. In some disulfide and ferredoxin reductases, the nicotinamide-binding site is occupied by the side-chain of an aromatic residue that protects FAD from the solvent in the ligand-free protein.\textsuperscript{40–43} The aromatic residue moves away upon binding of the pyridine nucleotide, allowing the nicotinamide

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{A proposed catalytic mechanism for the conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate. (i) In the substrate-binding pocket, the negative charge of the C4 carbon of the substrate is increased by formation of the hydrogen bonds with Asp75 and Tyr271 side-chains. (ii) The flavin reduced by NADPH reacts with an oxygen molecule in the protein interior, converting to the flavin C4a-hydroperoxide intermediate. (iii) One oxygen atom of the hydroperoxide is introduced into the C4 carbon on the aromatic ring by electrophilic aromatic substitution. (iv) The resulting oxonium cation intermediate is deprotonated, and then the product is released from the active site.}
\end{figure}
moiety to approach the isoalloxazine ring and to bind with the conformation required for hydride transfer.\textsuperscript{43} Presumably, the bulky side-chain of Tyr317 of MHBH interferes with NADPH binding so that Tyr317 could act as a “gatekeeper” controlling the contact of NADPH with the isoalloxazine ring.

The remarkable difference between the proposed mechanisms in PHHY and PHBH is whether the hydroxylation is required for substrate deprotonation.\textsuperscript{20,21} In PHBH, there is a proton-transfer network that is composed of Tyr201, Tyr385 and His72 (Figure 7(c)). This network has been shown to be responsible for the deprotonation of the substrate to enhance its nucleophilicity. The residues are not conserved in MHBH or PHHY (Figure 1(b)). Furthermore, it has been demonstrated that PHBH influences the acidity of the 4-hydroxyl group of the substrate by shifting the \( pK_a \) from 9.3 to 7.3, a physiological \( p\text{H} \) value, in order to carry out the deprotonation in the substrate-binding pocket.\textsuperscript{25–27} On the other hand, 3-hydroxybenzoate and phenol have the \( pK_a \) values 9.9 and 10.0, respectively, higher than that of 4-hydroxybenzoate.\textsuperscript{54} These facts may mean that MHBH and PHHY require the ability of substrate activation distinctly different from PHBH for the hydroxylation. In PHHY, the phenolate form of the substrate was not detected during its catalytic process.\textsuperscript{20} Because the active site of PHHY is almost identical with that of MHBH, it appears more probable that the catalytic process in MHBH is not mediated by the deprotonation of the substrate. Therefore, the protonated form of the substrate could suffer from the electrophilic attack by the flavin C4a-hydroperoxide intermediate, followed by the deprotonation of the resulting oxonium cation intermediate, as shown in Figure 9.

In the substrate-binding pocket of MHBH, the 3-hydroxyl group of the substrate forms direct hydrogen bonds with the side-chains of Asp75 and Tyr271 (Figure 5). The hydrogen bonds could allow the enzyme to transiently accept a proton from the hydroxyl group of the substrate, thereby increasing the acidity of the 3-hydroxyl group to some extent.\textsuperscript{20} Furthermore, the carboxyl group of the substrate is associated with the side-chains of His135 and Lys247 by two salt-bridges. The charge compensation by the positively charged residues would be required for stabilization of the carboxylate anion because the anion decreases the acidity of the hydroxyl group on the aromatic ring. In PHBH, a positively charged residue, Arg214, is used for charge compensation of the carboxylate anion of the substrate in a similar way (Figure 7(c)). Computational studies of PHBH have indicated that such electrostatic interactions are necessary to stabilize the negative charge of the carboxyl group and to decrease the oxidation barrier of the substrate toward the electrophilic aromatic attack by the flavin C4a-hydroperoxide intermediate.\textsuperscript{55–58} Thus, in MHBH, the electron-donating capacity of the 3-hydroxyl group of the substrate would be enhanced by the interactions with the surrounding residues in the substrate-binding pocket. Consequently, the C4 carbon atom to be hydroxylated would be activated sufficiently by the resonance effect of the hydroxyl group on the aromatic ring in order to suffer from the electrophilic attack by the reactive hydroperoxide intermediate.

In summary, the findings presented here provide insights into the structural basis for characterizing the substrate specificity of MHBH and help us understand its catalytic actions in molecular detail. One characteristic feature in the substrate-recognition mechanism is the presence of a large tunnel in the protein interior, which allows us to propose a pathway for substrate transport distinctly different from those of other flavoprotein aromatic hydroxylases of the same class. According to this transport mechanism, the potential substrate would be selected by the tunnel on the basis of its molecular size and amphiphilic nature. This hypothesis may imply that it is difficult to modify the substrate specificity by a rational design of the substrate-binding pocket. Structural studies with site-directed mutagenesis are required in order to investigate details of the substrate-recognition mechanism that could involve the activation of substrate.

**Materials and Methods**

**Expression of the MHBH protein in cells of *C. testosteroni* KH122-3s** was induced by the addition of 3-hydroxybenzoate and the protein was purified as described.\textsuperscript{5} Approximately 1 \( \mu \)g of purified protein was subjected to SDS-PAGE (10% polyacrylamide gel) and then electro-blotted onto a polyvinylidene fluoride membrane (Amerham Biosciences) by means of a Trans-Blot semi-dry transfer cell (Bio-Rad). A single band at the molecular mass of 71 kDa was excised from the membrane and the N-terminal amino-acid sequence, MQFLHNLGRGPNLIAPPAPLAPAH, was determined by Edman degradation.\textsuperscript{5} The isolated fragment was cleaved with endoproteinase Lys-C (Roche Diagnostics), and the internal amino-acid sequence MIDYEK was determined after purification of the proteolytic peptide fragments by a reverse-phase HPLC system at Biologica (Osaka, Japan).

DNA manipulations were carried out by standard methods.\textsuperscript{57} Bacterial chromosomal DNA was prepared from *C. testosteroni* cells by the sarkosyl method and purified by cesium chloride density-gradient centrifugation.\textsuperscript{60} In an attempt to obtain a partial DNA fragment of the MHBH coding region, PCR amplification was performed with the chromosomal DNA as a template and two degenerate primers. The upstream primer 5′-TTY TTY TCR TAR TCD ATN CCN AR-3′ was designed on the basis of the N-terminal internal amino-acid sequences, respectively. An amplified 1.8 kb fragment was used as a probe for Southern and colony hybridization to obtain the corresponding genomic clone. Digoxigenin labeling of the fragment, hybridization and detection were carried out according to the manufacturer’s instructions (DIG High Prime DNA Labeling and Detection Starter Kit I; Roche Diagnostics). Although digestion of the chromosomal DNA with PstI gave rise to...
a 4.0 kb fragment including the MHBH coding region, an attempt to clone it into the plasmid pUC19 (Takara Shuzo) using Escherichia coli TG-1 as a host strain failed due to the toxicity of the construct to the host cells. The PstI fragment was further digested with SpII and the resulting fragments were cloned into the plasmid pUC19. Three constructs containing different fragments, each with a piece of the gene encoding MHBH, were obtained. The nucleotide sequences were determined using a BigDye terminator cycle sequencing ready reaction kit, version 3.0 and a model 310 capillary DNA sequencer (Applied Biosystems), and analyzed with the DNASIS software package (Hitachi Software Engineering).

Crystallization and data collection

The purified protein was concentrated to approximately 10 mg ml$^{-1}$ in 25 mM phosphate buffer (pH 7.5) containing 0.3 mM 3-hydroxybenzoate. The protein solution was mixed with an equal volume of a reservoir solution consisting of 0.1 M 2-morpholinoethanesulfonate (Mes) (pH 6.5), 1.3 M ammonium sulfate, and 6% (v/v) 1,4-dioxane and crystals of MHBH in complex with its substrate were grown at 293 K by the sitting-drop, vapor-diffusion method. For structure determination, two kinds of mercury derivatives were prepared. The 4-chloromercuribenzoate (PCMB) derivative was obtained by co-crystallization in the reservoir solution containing 1 mM PCMB. The ethylmercurithiosalicylate (EMTS) derivative was prepared by soaking the substrate-complex crystals for 3 h in the reservoir solution containing 0.2 mM EMTS. Before data collection, all crystals were soaked for 1 min in the reservoir solution supplemented with 20% (v/v) glycerol and then flash-cooled in a nitrogen gas stream at 100 K. Diffraction data of the substrate complex crystal and the heavy-atom derivatives used for phasing were collected at beamline BL40B2 ($\lambda=1.000$ Å) at the SPring-8 (Harima, Japan) using a Quantum-4 CCD detector (ADSC). A high-resolution data set for the substrate complex crystal, with a maximum resolution of 1.8 Å, was collected at beamline BL44XU ($\lambda=0.900$ Å) at the SPring-8 using a DIP6040 imaging plate detector (Bruker AXS).

The Xe-derivative was prepared by exposing the substrate complex crystals to high-pressure xenon gas. The crystal, mounted on a loop, was exposed to xenon gas at 12 atm (1 atm = 101,325 Pa) for 1 h at room temperature in a CryoXe-Siter (Rigaku/MSC), then flash-cooled to cryogenic temperatures by injection into liquid carbon tetrafluoride. Diffraction data of the Xe-derivative crystal were collected at 100 K on a Quantum-4 CCD detector (ADSC) at beamline BL18B ($\lambda=1.000$ Å) at the Photon Factory (Tsukuba, Japan).

All diffraction data were indexed and integrated with MOSFLM, then further processed using programs from the CCP4 program suite. The data collection statistics are summarized in Table 1.

Structure determination and refinement

Initial phases for the substrate complex crystal were calculated from the MIRAS data sets. Two mercury atoms per asymmetric unit were identified for each derivative from the anomalous difference Patterson maps. The experimental phases were refined and calculated with MLPHARE. The resulting overall figure of merit was 0.55 at 3.0 Å resolution. Density modification with solvent flattening and phase extension to 2.8 Å in DM improved the quality of the initial electron-density map substantially and raised the figure of merit to 0.84. An initial model was built manually using the XtalView software package, then refined against the high-resolution data set for another substrate complex crystal by simulated annealing followed by refinement of individual $B$-factors in CNS. The cross-validated $\sigma_A$-weighted $2F_o-F_c$ and $F_o-F_c$ electron-density maps clearly revealed residual electron densities corresponding to the FAD and substrate molecules. Water molecules were added at positions of 3 $\sigma$ peaks in the electron-density maps located within hydrogen bonding distance of a donor or acceptor atom. After several rounds of iterative manual rebuilding, the substrate complex structure was refined at 1.8 Å to an $R_{work}$ of 17.3% and an $R_{free}$ of 19.8%. However, no electron density was found for residues 276–286 or 413–426 located on the protein surface. All main-chain angles were in the allowed regions of a Ramachandran plot, 90.6% in the most-favored regions, and showed excellent stereochemical quality. The Xe-derivative structure also was refined, resulting in an $R_{work}$ of 17.7% and an $R_{free}$ of 23.6%. A total of 87.9% of the amino acid residues were in the most-favored regions, except for Gln228, which showed unfavorable dihedral angles. The respective values of average $B$-factors were 35.0 Å$^2$ for the substrate complex structure and 31.8 Å$^2$ for the Xe-derivative structure. Statistics showing the results of phasing and structure refinement are given in Table 1.

Graphic images of molecular structure were generated by using the programs PyMOL\textsuperscript{‡}, MOLSCRIPT\textsuperscript{67} and Raster3D.\textsuperscript{68}

Data Bank accession codes

The nucleotide sequence of the MHBH coding region has been submitted to the DDBJ nucleotide sequence database under the accession number AB119008. The atomic coordinates and structure factors for the MHBH-substrate complex and Xe-derivative structures have been deposited in the RCSB Protein Data Bank with the respective accession codes of 2DKH and 2DKI.

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\textsuperscript{‡}http://pymol.sourceforge.net/
References


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