Biochemical and Biophysical Research Communications 371 (2008) 149–153

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Examination and expansion of the substrate range of *m*-hydroxybenzoate hydroxylase

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ARTICLE INFO

Article history:
Received 21 March 2008
Available online 14 April 2008

Keywords:
Comamonas testosteroni
m-Hydroxybenzoate hydroxylase
Directed evolution
Substrate specificity
Phenol
3-Aminophenol
Substituted catechol

ABSTRACT

The gene encoding *m*-hydroxybenzoate hydroxylase (*mobA*) was cloned from *Comamonas testosteroni* GZ39. MobA converts *m*-hydroxybenzoate and to a lesser extent *p*-hydroxybenzoate to protocatechuate. To explore the structural and functional relationships in phenolic acid monooxygenases, MobA was subjected to *in vitro* mutagenesis by error-prone PCR and the mutant MobAs were screened for their ability to oxidize phenol or 3-aminophenol. A mutant MobA with a single V257A substitution was able to transform phenol to catechol, providing the first example of monooxygenase acting on phenolic acids that can also hydroxylate phenol. The mutant MobA also has enhanced ability to transform resorcinol, hydroquinone, *p*-hydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, 3-chlorophenol, 4-chlorophenol, 4-chlororesorcinol, and 4-nitrophenol. Several MobA mutants were obtained for their ability to transform 3-aminophenol to a related substituted catechol. Mutant MobAs with single amino acid substitutions (H135P, A400G, or D416A) were derived from these mutants and verified for their ability to transform 3-aminophenol.

Microorganisms utilize aromatic hydroxylases (monooxygenases) to initiate the degradation of phenolic compounds [1]. Many aromatic hydroxylases have been studied extensively. For example, the *p*-hydroxybenzoate hydroxylase (PHBH; EC 1.14.13.2) from *Pseudomonas fluorescens* has become a "paradigm" of phenolic acid hydroxylase because its gene and crystal structure have been thoroughly examined [2]. Another well-known member of the family of aromatic hydroxylase is phenol hydroxylase (PHHY; EC 1.14.13.7) from the yeast *Trichosporon cutaneum*. The crystal structure of PHHY has revealed that the PHHY subunit consists of three domains [3]. The first two domains constitute the active site and bind the FAD cofactor and the phenolic substrate, while the C-terminal 200 residues fold into a separate domain that mediates subunit association. A comparison between the crystal structures of the first two domains of PHHY (664 amino acids) and PHBH (394 amino acids) shows high similarity even though their amino acid sequences have very little identity [4]. Although PHHY and PHBH catalyze similar reactions, no common substrates have so far been identified. PHHY has no activity towards phenols carrying carboxyl groups on the benzene nucleus or its side chain and PHBH has no activity towards phenol or substituted phenols. A question that has been raised is which sequence differences are responsible for the complete absence of cross-reactivity between PHHY and PHBH with respect to the aromatic substrates [4]. We chose to explore this question by using another enzyme—*m*-hydroxybenzoate hydroxylase (MobA) from *Comamonas testosteroni* GZ39, originally isolated from the Passaic River in New Jersey [5]. MobA attacks at the 4 position of *m*-hydroxybenzoate to produce protocatechuate (Fig. 1). The overall similarity between the MobA and PHHY is 53%, which is much higher than the similarity between PHBH and PHHY. However MobA, just like PHBH, lacks the ability to transform phenol.

The recently determined crystal structure of MobA from another *C. testosteroni* strain KH122-3s (98% identical to MobA from *C. testosteroni* GZ39) reveals a tunnel-like structure connecting the substrate-binding pocket to the protein surface, with possible functions involved in substrate selection and channeling [6]. The hypothesis however implies that it would be difficult to modify the substrate specificity by a rational design of the substrate-binding pocket [6]. In this study MobA was subjected to *in vitro* mutagenesis and subsequent *in vivo* screening for enzyme variants with altered substrate specificity. A mutant MobA with a single amino acid change is able to transform phenol and other substituted phenols with enhanced efficiency. This is the first example of a monooxygenase acting on phenolic acids that is also able to hydroxylate phenol. In a similar fashion MobA mutants capable of hydroxylating 3-aminophenol were also obtained.

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doi:10.1016/j.bbrc.2008.04.032

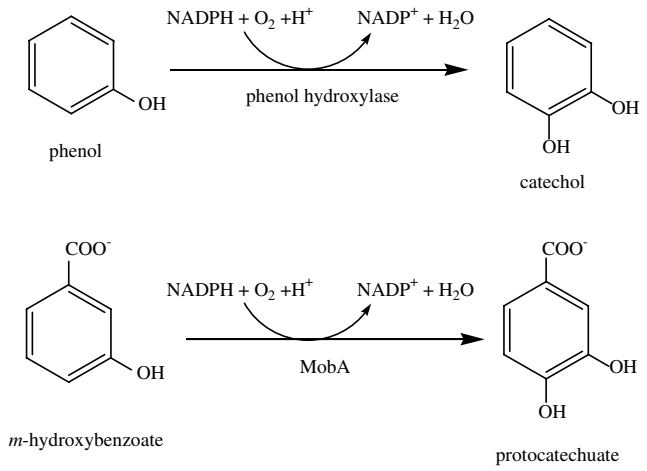

Fig. 1. Reactions catalyzed by phenol hydroxylase and *m*-hydroxybenzoate hydroxylase.

Materials and methods

Bacterial strains, media, and culture conditions. *Comamonas testosteroni* GZ39 was isolated from New Jersey for its ability to degrade phenanthrene [5]. *Escherichia coli* strains Top10, Top10F′, and DH5α (Invitrogen, Carlsbad, CA) were used in the cloning or the expression experiments and grown at 37 °C. LB medium and MSB medium were described in the previous study [7].

Molecular techniques. Plasmid DNA was purified using the NucleoSpin plasmid miniprep kit (Macherey-Nagel, Easton, PA). Restriction digests, ligations, transformations, and gel electrophoresis were performed following procedures as described before [7]. PCR was performed using a Perkin–Elmer GeneAmp PCR system 9700. *Taq* DNA polymerase, *Pfu* DNA polymerase and dNTPs were obtained from different sources. Oligonucleotides were purchased from Invitrogen (Carlsbad, CA) and their sequences are listed in Supplementary Table S1.

Screening cosmid clones for m-hydroxybenzoate hydroxylase activity. A cosmid library, previously constructed of *C. testosteroni* GZ39 [5] was screened for the *mobA* gene on MSB agar containing 5 mM glucose, 0.01% yeast extract, 0.01% casamino acids, 100 μg/ml ampicillin, 5 mM *m*-hydroxybenzoate, 50 μg/ml *p*-toluidine, and 1.5 mM ferric chloride [8]. Dihydroxylated products such as catechol and protocatechuate would react with the *p*-toluidine and ferric iron and develop a dark-brown color on the selective media [8].

Directed evolution of MobA. The *mobA* gene from pSEB2 was cloned into pZR80 [9] to produce pMobA_ZR80. The *mobA* gene was mutagenized by error-prone PCR using the GeneMorph PCR mutagenesis kit (Stratagene, La Jolla, CA). pMobA_ZR80 DNA (0.025 μg in 10 μl reaction) and the primers N1 and C3 were used in the reaction. This primer set amplifies the constitutive *tet* promoter along with the *mobA* gene. The blunt-ended PCR products were treated with *Taq* polymerase (72 °C, 15 min) before they were TOPO-cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The reaction mixture was transformed into *E. coli* Top10 and transformants were grown on LB plates containing 50 μg/ml kanamycin, 3 mM phenol, and 1.5 mM ferric chloride. Colonies hydroxylating phenol would turn black on this medium due to the complex formed by catechol and iron [8]. The mutant MobA with the ability to transform 3-aminophenol was created using the same strategy and selected on LB plates containing 50 μg/ml kanamycin, 2 mM 3-aminophenol, and 1.5 mM ferric chloride.

Constructing expression plasmids for the mutant MobAs. The plasmid for expressing $MobA_{WT}$ was constructed by amplifying the *mobA* gene along with the *tet* promoter from pMobA_ZR80 using *Pfu* DNA polymerase and the primer set N1-C3. The PCR product was cloned into pCR2.1-TOPO. The *tet* promoter area was then deleted from the plasmid by BamHI digestion. The expression of the *mobA* gene on the self-deletion plasmid (designated pMobA_WTB) is now under control of the IPTG inducible *lac* promoter. pMobA_V257A and pMobA_K326I were constructed as follows. pMobA_12D was digested by BamHI to produce pMobA_12DB. The 1.0-kb HindIII fragment containing the Val257 residue of pMobA_WTB was swapped with the HindIII fragment carrying the Val257Ala substitution of pMobA_12DB. The two new constructs have the Val257Ala and the Lys326Ile substitutions, respectively. The plasmids from the 3-aminophenol transforming clones were also purified and treated with BamHI to produce expression plasmids for the mutant MobAs. Two sequential PCRs were carried out to obtain the expression plasmids for $MobA_{Q292R}$, $MobA_{N227H}$, $MobA_{D416A}$, $MobA_{A400G}$, and $MobA_{K429R}$. Two PCR products from the 1st round PCRs (see Supplementary Table S2) were used as the templates for the 2nd round PCRs, which used primers 3AP-N and C3. After the 2nd round PCRs, the PCR products were cloned into pCR2.1-TOPO. The resulting plasmids were digested with BamHI to produce expression plasmids for the MobAs with the single amino acid substitution. For expressing $MobA_{N227H\text{-}D416A}$, the 2.1-kb SstI–XbaI fragment from the expression plasmid for $MobA_{D416A}$ was first transferred to pGEM-7Zf(-)- (Promega, Madison, WI). The 1.0-kb SstI–HindIII fragment of the new construct was replaced with the 1.0-kb SstI–HindIII fragment from the construct for expressing $MobA_{N227H}$. The 2.1-kb SstI–XbaI fragment of the resulting construct was transferred back to pCR2.1-TOPO for expressing $MobA_{N227H\text{-}D416A}$.

Substrate transformation. The *E. coli* Top10F′ strains carrying the expression plasmids were cultured on LB with 50 μg/ml kanamycin and 15 μg/ml tetracycline. The overnight culture was transferred to fresh media and cultured until the OD_{600} reached 0.5. IPTG was added at a final concentration of 1.0 mM to induce expression and the cells were harvested after 3 h by centrifugation. The collected cells were washed twice with phosphate buffer (50 mM sodium/potassium phosphate buffer, pH 6.8) and resuspended in the same buffer at an OD_{600} of 4.0. A reaction consisting of 6 ml of cells, 20 mM glucose, and 1.0 mM aromatic compound was placed in a 50 ml centrifuge tube and shaken at 200 rpm at 30 °C. Culture samples (1 ml) were taken at every 3 h after the compound was added. After centrifugation to remove the cells, the supernatant was filtered through a 0.45 μm Acrodisc® 13 mm syringe filter (Pall Gelman Laboratory, East Hills, NY) and analyzed by HPLC to determine the amount of the transformed compound [7]. Cell protein was determined by the method of Bradford [10]. The transformation rate was presented as the amount of substrate in nmol transformed by 1 mg of total protein per min. *E. coli* Top10F′ carrying pCR2.1-TOPO was used as a negative control and the loss of the compound in the background was subtracted from the calculated rates. The aromatic compounds used were obtained from Aldrich (Milwaukee, WI).

Sequencing. Sequencing reactions were performed as recommended by the supplier (Applied Biosystems, Inc.) and analyzed on an ABI 3100 automated DNA sequencer. The Accession number of the *mobA* gene from GZ39 is AY450844.

Results

Isolation and characterization of the gene for m-hydroxybenzoate hydroxylase from C. testosteroni GZ39

Five hundred fifty two cosmid clones from the cosmid library of *C. testosteroni* GZ39 were tested for *m*-hydroxybenzoate hydroxylase activity on the selective media containing *m*-hydroxybenzoate, *p*-toluidine, and ferric iron. One positive cosmid clone, designated pSEB2, was chosen for further analysis. The *mobA* gene was located in a 2.2-kb NsiI–KpnI fragment from pSEB2. The *mobA* gene encodes a 639 amino acid protein. It was confirmed that MobA converts *m*-hydroxybenzoate to protocatechuate by HPLC analysis of culture supernatants. RT-PCRs and a *mobA* knockout mutant constructed from *C. testosteroni* GZ39 further confirmed that the *mobA* gene is responsible for *m*-hydroxybenzoate metabolism and not for *p*-hydroxybenzoate metabolism (data not shown).

Directed evolution of MobA to hydroxylate phenol

Phenol is not the original substrate for wild-type MobA (designated $MobA_{WT}$) (Fig. 2). For directed evolution of MobA to accept phenol as a substrate, an expression plasmid for $MobA_{WT}$ (pMobA_ZR80) was first constructed. Random mutations were introduced into the *mobA* gene by error-prone PCR. The PCR products were then TOPO-cloned into pCR2.1-TOPO and the resulting plasmids were transformed into *E. coli* cells. The obtained clones were grown on LB plates containing phenol, ferric chloride, and kanamycin. The positive clones would form a dark color on the plates due to the complex formed by catechol and iron. One positive clone, 12D, was selected out of 600 clones screened. The ability of clone 12D to transform phenol to catechol was then confirmed by HPLC analysis. Sequencing of the plasmid pMobA_12D from clone 12D revealed that its *mobA* gene carried two mutations. One mutation changed the Val257 of the MobA to Ala257 and the other mutation changed the Lys326 to Ile326.

The V257A mutation enables the mutant MobA to transform phenol to catechol

Since the MobA from pMobA_12D has two mutations, it was necessary to construct separate clones with each mutation to identify the role of each amino acid substitution in the altered enzyme's

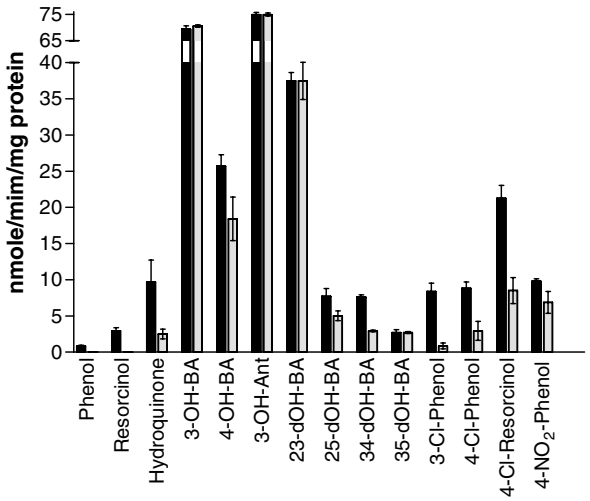

Fig. 2. Comparison of the ability of $MobA_{V257A}$ (black) and $MobA_{WT}$ (grey) to transform different compounds. *Abbreviations:* BA, benzoate; Ant, anthranilate; OH, hydroxy; dOH, dihydroxy; Cl, chloro; NO_2, nitro.

activity. Expression plasmids pMobA_V257A for producing MobA with the V257A mutation (designated $MobA_{V257A}$) and pMobA_K326I for producing the MobA with the K326I mutation (designated $MobA_{K326I}$) were constructed. The IPTG induced *E. coli* strain carrying either pMobA_V257A or pMobA_K326I was tested for its ability to transform phenol to catechol. Only the *E. coli* strain carrying pMobA_V257A could produce catechol from phenol. This result clearly demonstrates that $MobA_{V257A}$ is able to transform phenol to catechol while $MobA_{K326I}$, like $MobA_{WT}$ lacks this ability. The subtle change of valine to alanine at the position 257 alone enabled the mutant MobA to hydroxylate phenol to catechol.

The V257A mutation broadens the substrate range of the mutant MobA

$MobA_{V257A}$ and $MobA_{WT}$ expressed in *E. coli* were compared for their abilities to transform a variety of compounds in addition to phenol (Fig. 2). The tested compounds were added individually to IPTG-induced and concentrated *E. coli* cells producing $MobA_{V257A}$ or $MobA_{WT}$. The transformation reactions were carried out at 30 °C because both $MobA_{WT}$ and $MobA_{V257A}$ have higher enzymatic activity at 30 °C than at 37 °C (data not shown).

Neither $MobA_{WT}$ nor $MobA_{V257A}$ are capable of hydroxylating benzoate, *o*-hydroxybenzoate (salicylate), 2,4-dihydroxybenzoate, 2,6-dihydroxybenzoate, 2-chlorophenol, 3-aminophenol, 4-methoxybenzoate, 3-toluate, *o*-cresol, *m*-cresol, or *p*-cresol. Both $MobA_{WT}$ and $MobA_{V257}$ can transform hydroquinone, *m*-hydroxybenzoate, *p*-hydroxybenzoate (to a lesser extent than *m*-hydroxybenzoate), 3-hydroxyanthranilate, 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate (protocatechuate), 3,5-dihydroxybenzoate, 3-chlorophenol, 4-chlorophenol, 4-chlororesorcinol, and 4-nitrophenol. $MobA_{V257A}$ is able to transform hydroquinone, *p*-hydroxybenzoate, 2,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, 3-chlorophenol, 4-chlorophenol, 4-chlororesorcinol, and 4-nitrophenol with enhanced efficiency (Fig. 2). Only $MobA_{V257A}$ has the ability to transform phenol and resorcinol (Fig. 2). Products from the transformations were identified by HPLC analysis through comparison with known compounds' retention times and spectra. Hydroquinone was transformed to 1,2,4-benzenetriol. *p*-Hydroxybenzoate was transformed to protocatechuate (3,4-dihydroxybenzoate) and protocatechuate was further converted to gallic acid (3,4,5-trihydroxybenzoate). 3-Chlorophenol and 4-chlorophenol were both converted to 4-chlorocatechol. 4-Nitrophenol was transformed to 4-nitrocatechol. The products formed from 3-hydroxyanthranilate, 2,5-dihydroxybenzoate, and 4-chlororesorcinol were not identified. Phenol and resorcinol were transformed to catechol and 1,2,4-benzenetriol by $MobA_{V257A}$, respectively.

Directed evolution of MobA to hydroxylate 3-aminophenol

$MobA_{WT}$ is able to transform 3-substituted phenols such as 3-chlorophenol but is unable to hydroxylate 3-aminophenol. $MobA_{WT}$ was subjected to random mutagenesis and the MobA variants were screened on selective media containing 3-aminophenol. Ten clones (1-1, 1-3, 3-1, 6-1, 6-2, 11-1, 11-2, 13-1, 13-2, and 14-1) out of 6510 colonies screened formed a dark color on the selective medium. All these clones still retain the ability to transform *m*-hydroxybenzoate. Sequencing revealed that the MobA variants from these clones have a variety of amino acid substitutions. The mutant MobAs and their mutations are: $MobA_{1\text{-}1}$ (H135P), $MobA_{1\text{-}3}$ (R152L, F364V), $MobA_{3\text{-}1}$ (N227H, Q292R, D416A), $MobA_{6\text{-}1}$ (H135P), $MobA_{6\text{-}2}$ (F435Y, V479A), $MobA_{11\text{-}1}$ (H135P, I217L, Y304H), $MobA_{11\text{-}2}$ (A16T, S394P, D416A), $MobA_{13\text{-}1}$ (N102T, I259S, V399M), $MobA_{13\text{-}2}$ (H135P), and $MobA_{14\text{-}1}$ (A400G, K429R). $MobA_{1\text{-}1}$, $MobA_{6\text{-}1}$, and $MobA_{13\text{-}2}$ are identical to each other and contain the H135P mutation. The H135P mutation also appears in $MobA_{11\text{-}1}$. The D416A substitution is seen in $MobA_{3\text{-}1}$ and $MobA_{11\text{-}2}$. The mutant MobAs were compared for their ability to transform 3-aminophenol (Fig. 3). $MobA_{3\text{-}1}$ and $MobA_{14\text{-}1}$ have the highest enzymatic activity to hydroxylate 3-aminophenol.

Different amino acid substitutions allow MobAs to transform 3-aminophenol

$MobA_{3\text{-}1}$ and $MobA_{14\text{-}1}$ were examined further to determine which amino acid substitutions changed their substrate specificity. $MobA_{3\text{-}1}$ has the changes of Asn227 to His227, Gln292 to Arg292, and Asp416 to Ala416. $MobA_{14\text{-}1}$ has the changes of Ala400 to Gly400 and Lys429 to Arg429. The plasmids for expression $MobA_{N227H}$, $MobA_{Q292R}$, $MobA_{D416A}$, $MobA_{A400G}$, and $MobA_{K429R}$ were constructed (see Materials and methods). The new mutant MobAs were examined for their ability to hydroxylate 3-aminophenol (Fig. 3). $MobA_{D416A}$, derived from $MobA_{3\text{-}1}$, has the ability to transform

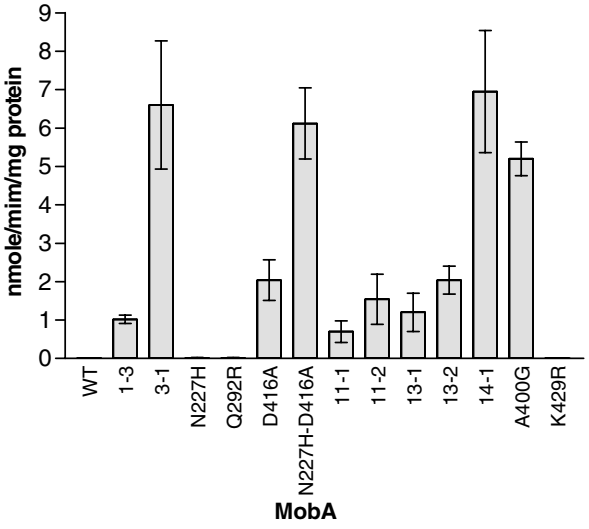

Fig. 3. Ability of different mutant MobAs to hydroxylate 3-aminophenol. The MobAs and their mutations are as followed: $MobA_{1\text{-}3}$ (R152L, F364V); $MobA_{3\text{-}1}$ (N227H, Q292R, D416A); $MobA_{11\text{-}1}$ (H135P, I217L, Y304H); $MobA_{11\text{-}2}$ (A16T, S394P, D416A); $MobA_{13\text{-}1}$ (N102T, I259S, V399M); $MobA_{13\text{-}2}$ (H135P); $MobA_{14\text{-}1}$ (A400G, K429R).

3-aminophenol, but with less efficiency than $MobA_{3-1}$. $MobA_{N227H}$ and $MobA_{Q292R}$ were not able to transform 3-aminophenol. However, $MobA_{N227H-D416A}$ almost has the same transformation efficiency as $MobA_{3-1}$. $MobA_{A400G}$ transformed 3-aminophenol with efficiency almost like $MobA_{14-1}$ while $MobA_{K429R}$ could not transform 3-aminophenol at all.

Discussion

More substrates for MobA were tested than the previous study on the MobA from *C. testosteroni* ATCC 17454 [11]. Most of the substrates of MobA contain a side chain with negative charge or polarity, which possibly interacts with the positively charged sidechains of Lys247 and His135 in the substrate-binding pocket (Fig. 4). The substrates also need a hydroxyl group to interact with Tyr271 and Asp75, as seen in the crystal structure of MobA [6] (Fig. 4). *m*-Cresol, *p*-cresol, 4-methoxybenzoate and 3-toluate cannot be substrates probably because of lacking such side chains. The position of the hydroxyl group is also important. It cannot be next to the group with negative charge or polarity unless there is another hydroxyl group at the proper position to interact with Tyr271 and Asp75. That is probably the reason why *o*-hydroxybenzoate, 2,6-dihydroxybenzoate, 2-chlorophenol cannot be the substrates and 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, 4-chlororesorcinol can be the substrates. *p*-Hydroxybenzoate and 3,4-dihydroxybenzoate, which have a hydroxyl group at the *para* position to the carboxyl group on their benzene rings, might have to turn to a position different from where *m*-hydroxybenzoate sits in the substrate-binding pocket in order to be attacked by oxygen at the correct carbon.

The Val257 to Ala257 mutation gives the MobA the new ability to hydroxylate phenol and resorcinol. This study provides the first example of a monooxygenase acting on phenolic acids that is also able to hydroxylate phenol. It seems that the V257A mutation might have a significant effect on its substrate binding. The distance of the side chain of Val257 to the substrate is close to 11 Å, measured using MDL Chime by Protein Explorer [12]. Val257 is surrounded by the hydrophobic side chains of Ile259, Phe270, Val272, and Val300. The closest non-hydrogen atom of the nearby residues to the gamma carbon No. 1 of Val257 is the nitrogen of Leu258 (3.0 Å). The closest non-hydrogen atom of the nearby residues to the gamma carbon No. 2 of Val257 is the oxygen of Asn256 (3.3 Å). Although Val257 is not a conserved amino acid residue among similar proteins, there are some highly conserved ones right after Val257: Ile260, Pro261, Arg262, Glu263, Arg269, and Tyr271. Tyr271 in MobA corresponds with Tyr289 in PHHY, which is important in orienting the substrate for attack at the *ortho* position by forming of a hydrogen bond with the hydroxyl moiety of the phenol [13]. The subtle change of Ile244 (corresponding with Tyr289) to Val244 in 2-hydroxybiphenyl 3-monooxygenase from *P. azelaica* HBP1 enables the enzyme to have a 30% higher specific activity with 2-*sec*-butylphenol, 2-methoxyphenol, and 2-hydroxybiphenyl [14]. Tyr271 in MobA forms hydrogen bonds with the isoalloxazine ring of the FAD molecule and the 3-hydroxyl group of the substrate [6]. The benzene ring of the substrate faces a hydrophobic surface formed by the sidechains of Leu258 and Ile260 [6]. It is possible that the V257A mutation has an influence on the neighboring amino acid residues that have direct contacts with the substrate (e.g. Tyr271 and Leu258). The disappearance of the gamma carbons of Val257 may change the steric positions Try271 and Leu258 relative to the substrate. This might explain why $MobA_{V257A}$ has higher activity than $MobA_{WT}$ on substrates such as *p*-hydroxybenzoate, 3,4-dihydroxybenzoate, 3-chlorophenol, 4-chlorophenol, 4-chlororesorcinol, and 4-nitrophenol, which might need to change their orientation in the substrate-binding pocket in order to be attacked by oxygen because of the *para* position of the hydroxyl group. The V257A mutation might make these compounds easier to move in the substrate-binding pocket.

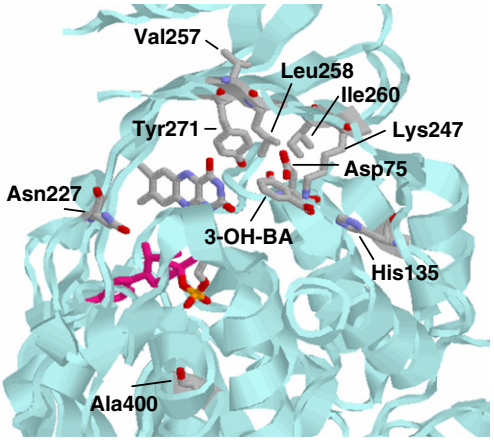

Fig. 4. Ribbon diagram of close-up view of the substrate-binding site of MobA. The *m*-hydroxybenzoate, FAD, and the amino acid residues are represented in stick models and colored by element. Asp416 is not shown here because its position in the crystal structure was not determined. The images were generated using the structural data of MobA from *C. testosteroni* strain KH122-3s (98% identical to MobA from *C. testosteroni* GZ39; Accession code 2DKH) and Protein Explorer 2.79 Beta [12].

The mutation sites of the 3-aminophenol transforming mutant MobAs are located in catalytic domain I and II, which are involved in FAD and substrate binding [6]. The proximity of His135 to the substrate-binding site makes it easier to explain why the H135P mutation alters the enzyme's substrate specificity. In the crystal structure, the side-chain of His135 forms a salt-bridge with the oxygen atom of the carboxyl group of *m*-hydroxybenzoate. 3-Aminophenol cannot be a substrate for $MobA_{WT}$ because its weak basic amino group cannot participate in such interactions. The H135P mutation which replaces the bulky and basic histidine residue with the nonpolar proline residue could make 3-aminophenol more acceptable in the substrate-binding pocket. Ala400, Asn227, and Asp416 just like Val257 are not in direct contact with the substrate. Ala400, located in helix structure H11, is at least 17 Å away from the substrate. Asn227, located in a flexible structure facing the surface, is about 14 Å away from the substrate. Asp416, located in an undefined area between helices H11 and H12 could be about 19 Å away from the substrate by judging from the structure of PHHY. Asn227 and Asp416 are located closer to the surface of the molecule than His135 and Ala400. It is interesting that helices H11 and H12 actually form part of the tunnel to the substrate-binding pocket in the protein interior [6]. For the A400G substitution, the change from alanine to glycine is relatively small. The D416A or N227H substitution is a much bigger change. The amino acid residue, which corresponds with the Ala400, is highly conserved in other enzymes including PHHY from *Trichosporon cutaneum*, pentachlorophenol 4-monooxygenases from *Sphingomonas* sp., and some putative hydroxylases. The amino acid residues, which correspond with the His135 and Asn227, can be found in some hydroxylases closely related to MobA, but not in PHHY or the pentachlorophenol 4-monooxygenases. The Asp416 is unique to MobA since it is not seen in the other enzymes for comparison. The assumed product, 4-aminocatechol, from the 3-aminophenol hydroxylation could not be detected probably because of the spontaneous air-oxidation of the compound [15].

This study gives us more insight into the nature of *m*-hydroxybenzoate hydroxylase. Only one location of the substitutions that changed the enzyme's substrate specificity dramatically is really predictable based on the information obtained from the crystal structure. Other substitutions are in the location, which would not be the targets for rational design for changing the enzyme's sub-

strate range. Some substitutions are also very subtle, reducing only one or two carbons in the side chains. Crystal structures of these MobA mutants might give more information about how these substitutions change the structure and hence the activity of the enzyme.

Acknowledgments

We thank Sarah E. Brandli and Anil K. Goyal for their contributions to the isolating *m*-hydroxybenzoate hydroxylase gene from *C. testosteroni* GZ39.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.032.

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