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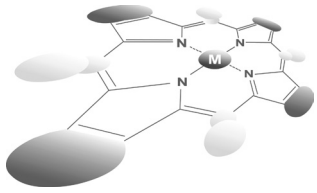

Selective hydroxylation of naphthalene using the H_2O_2-dependent engineered P450BM3 driven by dual-functional small molecule

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Dedicated to Professor Naisheng Chen on the occasion of his 80th birthday

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ABSTRACT: We herein report the H_2O_2-dependent selective hydroxylation of naphthalene catalyzed by engineered P450BM3 with the assistance of dual-functional small molecules (DFSMs). The mutation at position 268 significantly improved the hydroxylation activity of P450BM3, which is quite different from those engineered P450BM3 peroxygenases and NADPH-dependent P450BM3 mutants previously reported, implicating the unique role of the residue at position 268. This study provides a potential approach to develop the practical hydroxylation biocatalyst of P450s for aromatic hydrocarbons using the DFSM-facilitated P450BM3-H_2O_2 system.

KEYWORDS: P450 peroxygenase, hydrogen peroxide, hydroxylation, naphthalene, dual-functional small molecule.

INTRODUCTION

Selective hydroxylation of hydrocarbons is one of the most important transformations in organic synthesis. It is still a big challenge for chemical hydroxylation due to harsh reaction conditions, regioselectivity and reaction specificity. Enzymatic hydroxylation that is carried out under mild conditions with high selectivity is becoming a promising approach toward synthetic applications.

Among numerous oxygenases which can catalyze hydroxylation of hydrocarbons, cytochrome P450 monooxygenase is well known as the most versatile hydroxylase for various kinds of substrates such as alkanes, fatty acids, terpenes, and steroids, as well as aromatic compounds [1–6]. However, their practical application is challenged by their complicated electron transport chains and expensive cofactors such as NAD(P) H [7]. Using simple hydrogen peroxide (H_2O_2) is appealing due to its dramatically simplifying of the utility of P450 monooxygenases, but it is hampered by poor activity in the presence of H_2O_2. Until now, only a few native P450s are known to be capable of efficiently using H_2O_2 for hydroxylation or decarboxylation of fatty acids [8–9]. Many efforts have been devoted to developing man-made H_2O_2-dependent P450 systems [10–13].

Inspired by the catalytic mechanism of native peroxygenases for the activation of H_2O_2, we have recently developed a unique strategy for generating the peroxygenase activities of engineered cytochrome P450BM3 by the assistance of dual-functional small molecules (DFSMs), *N*-(ω-imidazolyl fatty acyl)-*L*-amino acid derivatives [14]. In this process, DFSM binds to P450BM3 by using the acyl amino acid moiety as an anchoring group, and the imidazolyl group of DFSM is used to mimic the general acid-base catalyst role of the key Glu196 residue in AaeAPO to trigger the activation of

◊SPP full member in good standing

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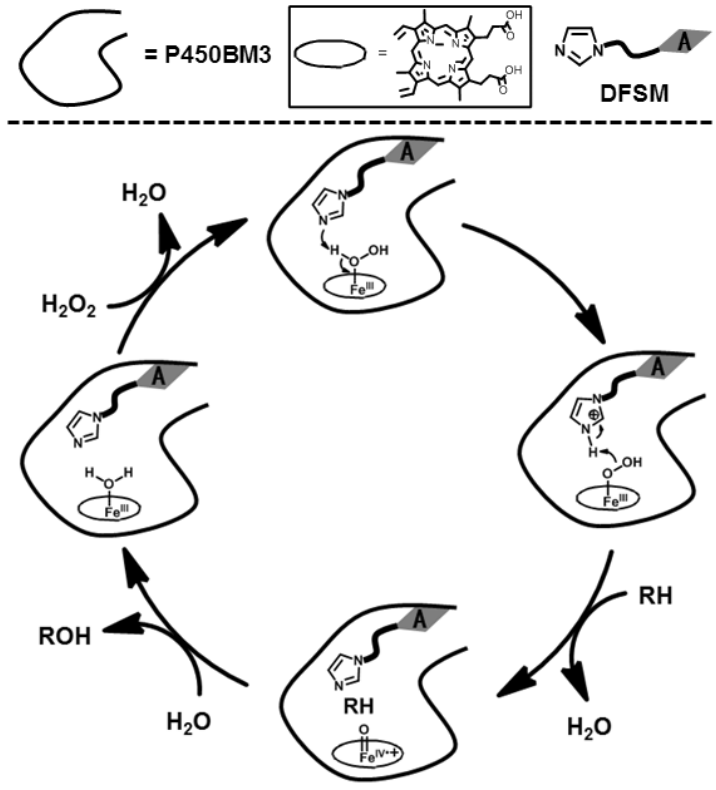

Fig. 1. The proposed catalytic cycle of the P450BM3-H_2O_2 system assisted by dual-functional small molecules (DFSMs) [14]

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H_2O_2 [15–16]. Subsequently, the active species Compound I is assumed to be formed [17], being responsible for the monooxygenation of non-native substrates (Fig. 1). This system has shown the best peroxygenase activity for the epoxidation of styrene, sulfoxidation of thioanisole, and benzylic hydroxylation of ethylbenzene among the reported P450-H_2O_2 systems to date. However, aromatic hydroxylation remains challenge for the man-made H_2O_2-dependent P450 peroxygenases. Encouraged by the fact that a small amount of 4-ethylphenol was detected in the hydroxylation of ethylbenzene, we envisioned that aromatic hydroxylation might be achieved by further improvement of the system through the engineering of P450BM3. As a result, a unique mutant F87G/T268V obtained by us yielded the H_2O_2-dependent hydroxylation activity of naphthalene assisted by the DFSMs, which does not share any mutation with previously reported engineered P450BM3 peroxygenases by other groups except for the mutation position of 87. Herein, we report the selective hydroxylation of naphthalene using the DFSM-facilitated P450BM3-H_2O_2 system.

EXPERIMENTAL

Chemicals and enzymes

All chemicals were purchased from commercial sources (*e.g.* Aldrich, TCI, Alladin, and J&K) and used without further purification unless otherwise noted. The DFSMs (Im-Cn-amino acids) were prepared as described previously (Fig. 2) [14]. The plasmid of cytochrome P450BM3 is a kind gift from Dr. Yujin Cao at Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences. Restriction endonucleases, T4 DNA ligase, Pfu DNA polymerase, IPTG, and SDS-PAGE protein ladder were obtained from Takara Co.

Mutagenesis, overexpression, and purification of P450BM3

All the F87 mutations were made by PCR-based site-directed mutagenesis and verified by DNA sequencing. The double mutants were located in the corresponding single mutant at position 87 background. The primers used for F87X were as previously reported [14]; for others were as follows: T268A-F: 5′-GCGACAAGTGG TCTTTTATCATTTGC-3′; T268I-F: 5′-ATCACAAGTGGTCT TTTATCATTTGC-3′; T268V-F; 5′-GTGACAAGTGGTCTTTT ATCATTTGC-3′; T268-R: 5′-TTCGTGTCCCGCAATTAAGA ATG-3′; A74G-F: 5′-GGTCTTAAATTTGTACGTGATTTTGC-3′; A74G-R: 5′-TTGACTTAAGTTTTTATCAAAGCGTG-3′; L188Q-F: 5′-CAGCAGCGAGCAAATCCAGACGA-3′; L188Q-R: 5′-CTTGTTCATTGCTTCATCCAGTGCAC-3′.

The overexpression and purification of cytochrome P450BM3 mutants were carried out as previously

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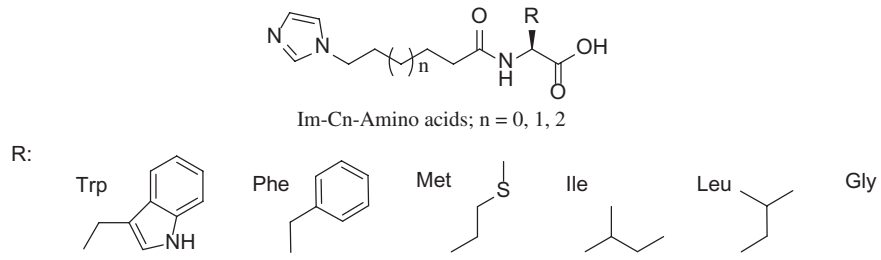

Fig. 2. The structures of DFSMs used in this study

described (Fig. S1) [14]. Formation of a ferrous CO complex was confirmed by UV-vis spectral change through the reduction of ferric heme of the wild type P450 or its mutants by addition of $Na_2S_2O_4$ in the presence of carbon monoxide (CO) (Fig. S2). The concentrations of P450s were measured from the CO-difference spectra according to the reported method by using ε = 91 $mM^{-1} \cdot cm^{-1}$ [18].

Reaction condition

Typical reaction mixtures (1 mL) contained purified P450BM3 enzymes (0.5 μM), 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, Im-C6-Phe (500 μM), DMSO (2% vol/vol), and naphthalene (1 mM). The reaction was initiated by the addition of H_2O_2 (20 mM). The reaction mixture was incubated in a water bath at 25 °C for 30 min. The reaction was stopped by the addition of dilute aqueous HCl. The reaction mixture was neutralized and extracted with 1 mL of ethyl acetate, and the organic phase was separated and dried with anhydrous sodium sulphate.

Product analysis

The products were analyzed by gas chromatography using a Shimadzu GC-2010 Plus gas chromatograph with a flame ionization detector (FID) and fitted with an AOC-20i auto sampler system using a DB-5 column (length: 30 m, internal diameter: 0.25 mm, film thickness: 1.0 μm, Agilent, USA). The analytical conditions were as follows: splitting ratio: 1/9, temperature program: injector 280 °C, detector 300 °C, 100 °C oven for 1 min, then 15 °C/min gradient to 200 °C, 60 °C/min gradient to 280 °C and then 280 °C for 4 min (total 13 min). 1-naphthol was identified according to an authentic sample. The yield of 1-naphthol was estimated using benzophenone as an internal standard.

RESULTS AND DISCUSSION

The UV-vis spectra of the purified P450BM3 mutants showed an absorption maximum at 417 nm that was identical to the typical low-spin water-bound P450 heme spectrum except for F87A/T268I, F87G/T268V, and F87V/T268V (Fig. S2). The last three exhibited spectra indicate a mixture of low- and high-spin forms. This may be the replacement of Thr268 with the hydrophobic residues disturbed the sixth axial ligand of water molecules of iron heme [19]. The reduced forms of F87A/T268I and F87G/T268V displayed shoulder peaks at around 420 nm, which maybe ascribe to the different mode of cysteine ligation [20]. At any rate, the altered spectral features didn't affect the peroxygenase activity of the mutants. The result is similar to that observed in a natural H_2O_2-dependent P450 decarboxylase [21].

In view of the critical effect of the residue size at position 87 in cytochrome P450BM3 on the H_2O_2-supported hydroxylation activity of long-chain fatty acids [22], the hydroxylation of naphthalene was first examined in the presence of H_2O_2 (20 mM) and Im-C6-Phe (500 μM) [14] in 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl by using the heme domain of the wild-type P450BM3 and its single mutants including F87G, F87A, F87V, F87I, and F87L. As a result, only F87G and F87A yielded 1-naphthol in a catalytic turnover number (TON) of 19 and 13, respectively (Table 1, Fig. 3). On the other hand, although it has been reported that F87A supports low levels of peroxygenase activity for epoxidation of styrene, sulfoxidation of thioanisole, sp^3 C–H hydroxylation of ethylbenzene and fatty acids [14, 22–23], no oxidized products were detected without Im-C6-Phe in the current reaction. These results suggest that DFSM plays an important role in producing hydroxylation activity of naphthalene. In addition, the free space size in the vicinity of the heme iron still influences the reaction activity for the DFSM-facilitated P450BM3-H_2O_2 system [14].

A previous report has shown that the double mutant of P450BM3 F87A/T268V efficiently oxidized ethylbenzene to yield 1-phenylethanol (85%), 2-phenylethanol (11%), and 4-ethylphenol (4%) in a total TON of 373 in the presence of H_2O_2 and Im-C6-Phe [14], while the combination of F87A and Im-C6-Phe only yielded 1-phenylethanol (92%) and 2-phenylethanol (8%) in a total TON of 39 in H_2O_2 (unpublished data). We envisioned that the residue at position 268 should play a key role in the generation of hydroxylation activity by the DFSM-facilitated P450BM3-H_2O_2 system. Thus, the H_2O_2-dependent hydroxylation of

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Table 1. H_2O_2-dependent hydroxylation of naphthalene catalyzed by P450BM3 mutants in the presence of dual-functional small molecules

Entry	P450BM3	DFSM	H_2O_2/mM	TON[a]
1	WT	Im-C6-Phe	20	—
2	F87G	Im-C6-Phe	20	19
3	F87A	Im-C6-Phe	20	13
4	F87A/T268V	Im-C6-Phe	20	41
5	F87A/T268A	Im-C6-Phe	20	7
6	F87A/T268I	Im-C6-Phe	20	8
7	F87V/T268V	Im-C6-Phe	20	8
8	F87G/T268V	Im-C6-Phe	20	144
9	F87G/T268V	Im-C6-Phe	60	203
10	F87G/T268V	Im-C5-Phe	60	94
11	F87G/T268V	Im-C6-Gly	60	13
12	F87G/T268V	Im-C6-Leu	60	15
13	F87G/T268V	Im-C6-Ile	60	60
14	F87G/T268V	Im-C6-Trp	60	24
15	F87G/T268V	Im-C6-Met	60	19
16	A74G/F87A	Im-C6-Phe	20	24
17	F87A/L188Q	Im-C6-Phe	20	9

[a] TON: Turnover numbers were calculated for three parallel experiments with the standard deviations less than 20%.

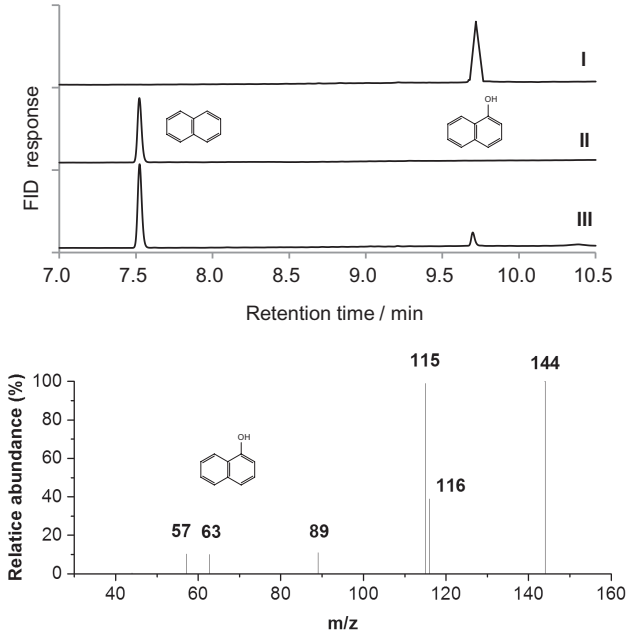

Fig. 3. (Top) GC analysis for the formation of 1-naphthol from the hydroxylation of naphthalene catalyzed by F87G/T268V with the assistance of Im-C6-Phe in H_2O_2 in 20 mM Tris-HCl buffer (pH 7.4) at 25 °C, authentic sample (I); control without Im-C6-Phe (II); complete reaction (III). (Bottom) Mass spectrum showing molecular ions of 1-naphthol

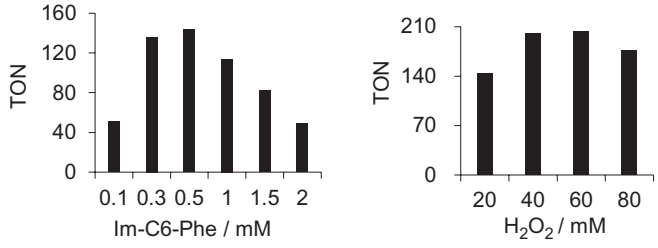

Fig. 4. Optimization of concentrations of Im-C6-Phe (Left) and H_2O_2 (Right) for the hydroxylation of naphthalene catalyzed by F87G/T268V (0.5 μM) at 25 °C

naphthalene was examined by F87A/T268V to give 1-naphthol in a TON of 41 (Table 1). We then examined the effect of the residue size at position 268 on the hydroxylation of naphthalene. Either F87A/T268A or F87A/T268I yielded lower TONs for the formation of 1-naphthol (Table 1). However, the mutants F87G/T268V and F87V/T268V were examined to find that the former showed the best activity in a TON of 144 among all the P450BM3 mutants used. 1-naphthol was the sole product according to the analysis of GC and GC-MS (Fig. 3).

The hydroxylation of naphthalene by F87G/T268V was then optimized under various concentrations of Im-C6-Phe and H_2O_2 (Fig. 4). The reaction gave a TON of 144 in the presence of 0.5 mM Im-C6-Phe in 20 mM H_2O_2 at 25 °C. The best TON of 203 was obtained when the reaction was carried out in H_2O_2 (60 mM) in the presence of Im-C6-Phe (0.5 mM).

The chain length of DFSMs significantly influences the hydroxylation of naphthalene catalyzed by F87G/T268V. Indeed, Im-C7-Phe didn't yield any oxidized products (data not shown) but Im-C5-Phe gave a TON of 94 for 1-naphthol (Table 1). These results are consistent with the previous report, in which the length of DFSM was thought to be a critical factor for the activation of H_2O_2 in the DFSM-facilitated P450BM3-H_2O_2 system [14].

The effect of the DFSM anchoring group was also examined for the hydroxylation of naphthalene catalyzed by F87G/T268V. Only Im-C6-Leu yielded a moderate TON of 60, while other DFSMs showed lower TONs (Table 1, Entries 11–15). According to previous reports, the various acyl amino acid groups mainly determined the binding affinity of DFSMs for P450BM3 [14, 24–25], which may be directly related to the ability of DFSM to activate H_2O_2.

Although previous studies revealed that NADPH-dependent P450BM3 variants (such as P450BM3_F87V) could hydroxylate naphthalenes [26–27], to the best of our knowledge, there were no reports of the H_2O_2-dependent hydroxylation of naphthalene catalyzed by the engineered P450BM3 peroxygenase [7, 9, 13–14, 28]. This is in good agreement with our findings as shown in

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the current study. DFSMs play a critical role in generating peroxygenase activity of P450BM3 for naphthalene hydroxylation as we expected. However, to our surprise, the substitution of Thr268 with Val268 in the F87A mutant of P450BM3 resulted in 3-fold improvement in the catalytic TON. This is quite different from the results we observed in the epoxidation of styrene by the DFSM-facilitated P450BM3-H_2O_2 system [14]. In that case, the TON of F87A/T268V was almost 4 times lower than that of F87A in the presence of Im-C6-Phe. That result is easier to understand because Thr268 is well known to play a role in the formation of active species compound I in the catalytic cycle of P450BM3 [5, 29–30]. We have also indirectly confirmed this possible function of Thr268 due to the complete loss of peroxygenase activity of the F87A/T268V mutant of P450BM3 in the absence of DFSMs. The reason why the hydroxylation activity of naphthalene is improved by the introduced Val268 is still unclear. One possible explanation is that the combination of mutations at positions 268 and 87 efficiently adjusts the active site pocket size to accommodate the naphthalene substrate. Indeed, the TON of F87G/T268V was further improved 11-fold over F87A as observed in this study. It has ever been reported that the mutation of Ala74 and Leu188 together with Phe87 dramatically improved the oxidation of polycyclic aromatic hydrocarbons including naphthalene in the NADPH-dependent P450BM3 system [26–27]. Accordingly, we prepared the mutants A74G/F87A and F87A/L188Q to examine the hydroxylation activity of naphthene using the current H_2O_2-system. However, comparable TONs were observed only for these two mutants in comparison with F87A (Table 1). These results partially supported the assertion that the residue at position 268 played a unique role in the hydroxylation activity of naphthalene by the DFSM-facilitated P450BM3-H_2O_2 system.

CONCLUSION

In summary, we have shown for the first time that the engineered P450BM3 could catalyze the H_2O_2-dependent hydroxylation of naphthalene with the assistance of DFSMs. This study increases the potential of cytochrome P450s for developing the practical hydroxylation biocatalysts using the DFSM-facilitated P450-H_2O_2 system by the avoidance of their complicated electron transport chains. The mutation of the residue at position 268 unexpectedly improved the H_2O_2-dependent hydroxylation activity in the current system. The unique role of Val268 may be further understood with the help of X-ray structure analysis, molecular dynamics simulations, and theoretical calculations. Such work and further studies aimed at improving the hydroxylation activity of aromatic hydrocarbons including but not limited to naphthalene are in progress by combining additional mutations in the DFSM-facilitated P450-H_2O_2 system.

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Supporting information

Figures S1 and S2 are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

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