Direct Hydroxylation of Benzene to Phenol by Cytochrome P450BM3 Triggered by Amino Acid Derivatives

Osami Shoji,[b] [c]* Sota Yanagisawa,[b] Joshua Kyle Stanfield,[b] Kazuto Suzuki,[b] Zhiqi Cong,[b] Hiroshi Sugimoto,[c] [d] Yoshitsugu Shiro,[d] and Yoshihito Watanabe [a]*

Abstract: Selective hydroxylation of benzene to phenol, without formation of side products resulting from overoxidation, was catalyzed by cytochrome P450BM3 (P450BM3) with the assistance of amino acid derivatives as decoy molecules. The catalytic turnover rate and the total turnover number reached 259 $min^{-1}P450BM3^{-1}$ and 40,200 $P450BM3^{-1}$ when *N*-heptyl-L-proline modified with L-phenyl alanine (C7-L-Pro-L-Phe) was used as the decoy molecule. This work shows that amino acid derivatives that have a totally different structure from fatty acids can be used as decoy molecules for aromatic hydroxylation by wild-type P450BM3. This methodology for nonnative substrate hydroxylation by wild-type P450BM3 has the potential to expand the utility of P450BM3 for biotransformations.

Enzymes catalyze a wide variety of reactions under very mild conditions, and have therefore been regarded as promising green catalysts for producing pharmaceuticals, fine chemicals, and biofuels. Developing a catalyst for selective oxyfunctionalizations is a longstanding challenge and a current topic of interest.[1] Cytochrome P450s (P450s) are regarded as potential candidates for the development of biocatalysts because of their high catalytic activity in the hydroxylation of unactivated C–H bonds.[2] A plethora of engineered P450s have thus been constructed by mutagenesis and chemical modifications.[3] Among the reported P450s, CYP102A1 (P450BM3) isolated from *Bacillus megaterium* has garnered much attention because of its high monooxygenase activity.[4] In general, P450BM3 displays a high substrate specificity, exclusively catalyzing the hydroxylation of long-alkyl-chain fatty acids (Fig. 1a) while remaining inactive for small non-native substrates such as propane and benzene. However, it was observed that P450BM3 can be “fooled” into initiating hydroxylation of non-native substrates in the presence of perfluorinated carboxylic acids (PFCs), which function as inert dummy substrates (decoy molecules).[5] [6] These PFCs mimic the native fatty acyl substrate and are capable of erroneously inducing the activation of molecular oxygen and subsequent generation of compound I,[7] which due to the inert nature of the C–F bonds (C–F bond energy)[8] of the PFC leads to the hydroxylation of gaseous alkanes and benzene (Fig. 1b). Considering that alkyl carboxylic acids with shorter alkyl chains, such as nonanoic acid (C9), cannot serve as decoy molecules, it was concluded that fluorination of carboxylic acids is critical for decoy molecule function.

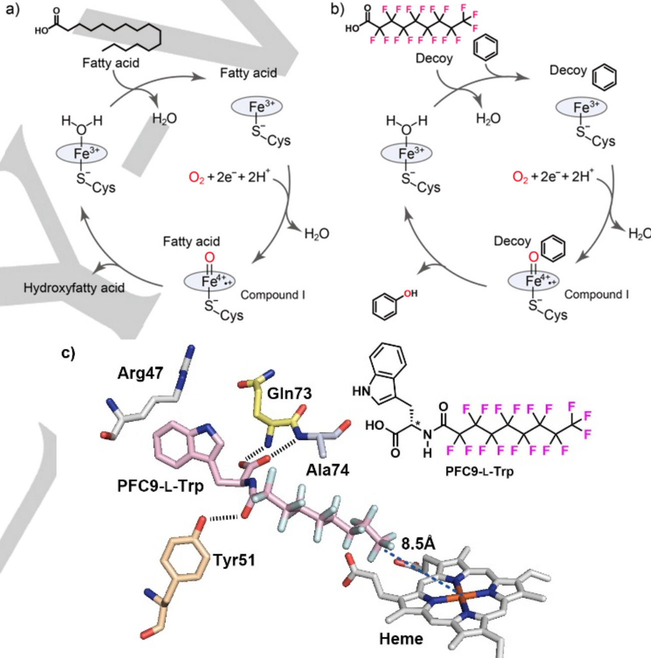

Figure 1. a) General reaction mechanism of fatty acid hydroxylation catalyzed by P450BM3. b) A plausible reaction mechanism of benzene hydroxylation catalyzed by P450BM3 with perfluorononanoic acid (PFC9) as a decoy molecule. c) The active site structure of P450BM3 with *N*-perfluorononanoyl-L-tryptophan (PFC9-L-Trp). The distance between the heme iron atom and the terminal carbon atom of PFC9-L-Trp is shown by a blue dashed line.

Recently, we succeeded in enhancing the catalytic activity of P450BM3 for gaseous alkanes by developing a series of second-generation decoy molecules, namely *N*-perfluoroacyl amino acids.[9] For example, propane was efficiently hydroxylated by P450BM3 with the assistance of *N*-perfluorononanoyl-L-leucine (PFC9-L-Leu). Crystal structure analysis of the *N*-perfluorononanoyl-L-tryptophan (PFC9-L-Trp)-bound form of P450BM3 (PDB code: 3WSP) revealed that the alkyl chain terminus does not fully penetrate into the active site owing to multiple hydrogen-bonding interactions between the carboxyl and carbonyl groups of PFC9-L-Trp and the amino acids (Tyr-51, Gln-73, and Ala-74) located at the entrance of the active site of P450BM3 (Fig. 1c). Therefore, we hypothesized that the alkyl chain terminus of nonanoic acid (C9) modified with tryptophan

[a] Prof. Dr. Y. Watanabe
Research Center for Materials Science, Nagoya University
Furo-cho, Chikusa-ku, Nagoya, 464-8602 (Japan)
Fax: (+81) 52-789-2953
E-mail: p47297a@nucc.cc.nagoya-u.ac.jp
[b] Dr. O. Shoji, S. Yanagisawa, J. K. Stanfield, K. Suzuki, Z. Cong
Department of Chemistry, Graduate School of Science
Nagoya University
Furo-cho, Chikusa-ku, Nagoya, 464-8602 (Japan)
Fax: (+81) 52-789-3557
E-mail: shoji.osami@a.mbox.nagoya-u.ac.jp
[c] Dr. O. Shoji, Dr. H. Sugimoto
Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 5 Sanbancho, Chiyoda-ku, Tokyo, 102-0075, Japan
[d] Prof. Dr. Y. Shiro, Dr. H. Sugimoto
RIKEN SPring-8 Center
Harima Institute
1-1-1 Kouto, Sayo, Hyogo 679-5148 (Japan)

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(C9-L-Trp) would be sufficiently distant from the heme iron to prevent hydroxylation by P450BM3, even in the absence of fluorination. If the hydroxylation of carboxylic acids were to be suppressed by modification with amino acids, diverse carboxylic acids without the requirement for fluorination could be exploited as building blocks for novel decoy molecules. Herein, we report that various carboxylic acids modified with amino acids (*N*-acyl amino acids) as well as amino acid dipeptides (Fig. 2) can serve as decoy molecules for P450BM3. Benzene was more efficiently hydroxylated in the presence of *N*-heptyl-L-proline modified with L-phenylalanine (C7-L-Pro-L-Phe, Fig. 2) than with any other decoy molecule. The maximum turnover rate and coupling efficiency ([phenol]/[NADPH consumption]×100) were 259 min^{-1}P450BM3^{-1} and 43%, respectively, and the total turnover number (TON) reached 40,200 ± 1,700 P450^{-1}.

PFC9 C9 R: Ala $-CH_3$ Met Leu Phe Trp PFC9-L-Amino acid C(n+7)-L-Amino acid n=1, 2, 3, 4 C9-D-Amino acid (*R* and *S*)-Ibu-L-Phe Z-L-Pro-L-Phe Ph-C6-L-Phe Z-Gly-L-Phe C7-L-Pro-L-Phe

Figure 2. Structure of decoy molecules.

Initially, we examined the possibility of using nonanoic acid modified with tryptophan (C9-L-Trp) and leucine (C9-L-Leu) as decoy molecules for benzene hydroxylation. In the presence of these decoy molecules, benzene was hydroxylated to phenol without formation of any side products such as catechol and hydroquinone resulting from overoxidation of phenol, (Table 1 and Fig. S1), while no product was detected when bare nonanoic acid (C9) was used as a decoy molecule. The catalytic turnover rate of phenol formation reached 169 min^{-1}P450^{-1} in the case of C9-L-Trp, which was higher than that for perfluorinated PFC9-L-Trp at 134 min^{-1}P450^{-1} (Table 1). These results clearly indicate that the simple modification of the nonanoic acid carboxylate moiety with tryptophan is sufficient to convert it into an effective decoy molecule. Furthermore, these findings also show that the previous assumption that fluorination is required for decoy molecule function in the case of P450BM3 can be dismissed. To investigate the effect of the amino acid side chain, a series of nonanoic acid derivatives modified with amino acids (C9-L-amino acids) were prepared. The catalytic turnover rate was highly dependent upon the type of amino acid modification, and C9 derivatives modified with L-amino acids possessing larger side chains displayed a tendency to exhibit higher catalytic activities. C9-L-Phe afforded the highest turnover rate (192 min^{-1}) of all C9-L-amino acids examined in this study. It is important to note here that modification with L-amino acids (C9-L-Phe and C9-L-Leu) was superior to modification with D-amino acids (C9-D-Phe and C9-D-Leu), indicating that amino acid chirality also affects catalytic activity. Catalytic activity was also dependent upon the decoy molecule's alkyl chain length, with C9-L-Phe and C10-L-Phe delivering better turnover rates than longer or shorter variants in this series. The UV-Vis spectral perturbations of P450BM3 recorded upon the addition of *N*-acyl amino acids were very small (Fig. S2), indicating a lack of a clear spin transition of the heme iron, rendering the determination of reliable dissociation constants (K_d) for *N*-acyl amino acids by UV-Vis titration experiment challenging. We tentatively estimated K_d values for binding of *N*-acyl amino acids from the very small observed spectral perturbations obtained (Fig. S2); however, no decisive differences could be observed, with all K_d values clustered in a similar range (8–41 μM). Although the UV-Vis spectral perturbations upon addition of *N*-acyl amino acids were not clear, we presumed that *N*-acyl amino acids bind to the active site in a similar manner to that observed with PFC9-L-Trp (Fig. 1c). This assumption is supported by reports describing the non-native substrates propranolol and propylbenzene, for which the spin transition of the

Table 1. Turnover rate and coupling efficiency of benzene hydroxylation[a]

P450BM3, Decoy molecule; O_2 + NADPH; OH

Decoy molecule	Rate [/min/P450][b]	Coupling efficiency[c] [%]
PFC9	120 ± 5 Ref. [6b]	19
PFC9-L-Trp	134 ± 9	29
PFC9-L-Phe	157 ± 4	31
Nonanoic acid (C9)	n.d.	n.d.
C9-L-Trp	169 ± 10	41
C9-Gly	9 ± 1	11
C9-L-Ala	12 ± 1	8
C9-L-Met	90 ± 5	39
C9-L-Leu	60 ± 6	39
C9-D-Leu	9 ± 1	13
C9-L-Phe	192 ± 8	41
C9-D-Phe	104 ± 6	38
C8-L-Phe	38 ± 3	20
C10-L-Phe	201 ± 11	44
C11-L-Phe	175 ± 7	45
Ph-C6-L-Phe	51 ± 4	19
R-Ibu-L-Phe	225 ± 9	36
S-Ibu-L-Phe	236 ± 4	40
Z-Gly-L-Phe	64 ± 2	47
Z-L-Pro-L-Phe	229 ± 9	41
C7-L-Pro-L-Phe	259 ± 12	43

[a] Reaction conditions: P450BM3 (0.5 μM), decoy molecule (100 μM), benzene (10 mM), NADPH (5 mM). [b] Uncertainty is given as the standard deviation of at least three measurements. [c] ([Phenol]/[NADPH consumption])×100. n.d. not detected.

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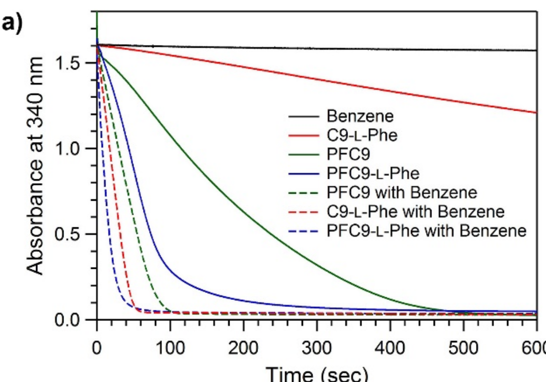
heme iron of P450BM3 upon addition of these substrates was also ill-defined, despite their being oxidized by P450BM3.[10] Consistent with the small spectral perturbations seen upon addition of *N*-acyl amino acids, the consumption of NADPH in the presence of C9-L-Phe alone was significantly slower than with PFC9-L-Phe. However, NADPH was consumed rapidly when benzene was added simultaneously (Fig. 3a). These results clearly showed that C9-L-Phe alone was not sufficient to activate P450BM3, but that P450BM3 was fully activated in the presence of both C9-L-Phe and benzene. This may be one reason for the increased coupling efficiency of C9-L-Phe (41%) compared with that of PFC9-L-Phe (29%). Formation of the CO adduct (CO-Fe^{2+}) using a CO-saturated buffer was also accelerated in the presence of both C9-L-Phe and benzene (Fig. 3b), indicating that heme reduction from Fe^{3+} to Fe^{2+} was synergistically accelerated by both decoy molecule and benzene binding to the active site simultaneously.



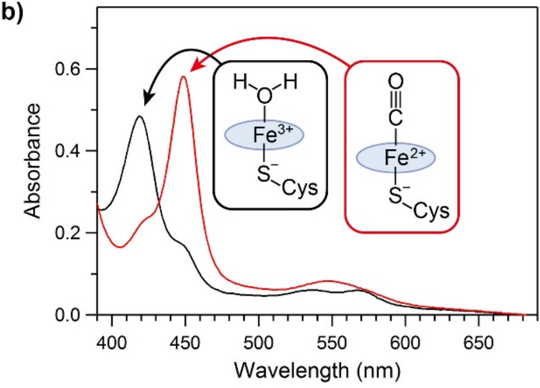


Figure 3. a) Time course measurement of NADPH consumption by P450BM3 with 100 μM decoy molecules (PFC9 (green), PFC9-L-Phe (blue), and C9-L-Phe (red)) in the absence (solid line) and presence (dashed line) of 10 mM benzene monitored by absorption at 340 nm. The concentrations of P450BM3 and NADPH were 0.25 μM and 250 μM, respectively. As a control experiment, the same amount of dimethyl sulfoxide was added to the reaction without decoy molecule (black line). b) UV-Vis spectra of P450BM3 in a CO-saturated buffer with 250 μM NADPH and 100 μM C9-L-Phe before (black line) and after (red line) the addition of 10 mM benzene.

Given that a myriad of nonfluorinated carboxylic acids can be employed as building blocks for decoy molecules, we prepared different types of decoy molecules possessing a phenyl group using carboxylic acids with a length equivalent to C10-L-Phe. We expected that a potential π–π stacking interaction between benzene and the phenyl ring of the decoy molecules would improve the turnover rate of benzene hydroxylation. Amino acid dipeptides containing a carboxybenzyl protecting group (Z-protection group) were also prepared as potential decoy molecules. Interestingly, ibuprofen modified with phenylalanine (*R* and *S*-Ibu-L-Phe) and Z-L-Pro-L-Phe showed relatively increased catalytic activities (Table 1), while Z-Gly-L-Phe and Ph-C6-L-Phe were subpar. We also examined the hydroxylation of toluene and anisole by P450BM3, employing *S*-Ibu-L-Phe as the decoy molecule (Table 2), wherein the *ortho*-position of monosubstituted benzenes was selectively hydroxylated. The turnover rates for hydroxylation of toluene and anisole were 641 and 623 min^{-1}P450^{-1} with high coupling efficiencies (62% and 58%). This coupling efficiency was superior to that of the engineered P450BM3 variant (R47S/Y51W/I401M, 48% for toluene and 44% for anisole), albeit the initial turnover rate was inferior to that of the mutant.[11] The turnover rate for aromatic hydroxylation of monosubstituted benzenes with *S*-Ibu-L-Phe exceeded that of PFC9 (220 min^{-1}P450^{-1} for toluene, 260 min^{-1}P450^{-1} for anisole).[6b]

Table 2. Turnover rate and coupling efficiency of hydroxylation of toluene and anisole using *S*-Ibu-L-Phe as the decoy molecule[a]

Substrate	Rate [min^{-1}P450^{-1}][b]	Coupling efficiency[c] [%]	*Ortho*-selectivity [%]
Toluene ($Ph\text{-}CH_3$)	641 ± 22	62	96
Anisole ($Ph\text{-}OCH_3$)	623 ± 27	58	92

[a] Reaction conditions: P450BM3 (0.5 μM), *S*-Ibu-L-Phe (100 μM), benzene (10 mM), NADPH (5 mM). [b] Uncertainty is given as the standard deviation of at least three measurements. [c] ([Phenol]/[NADPH consumption])×100.

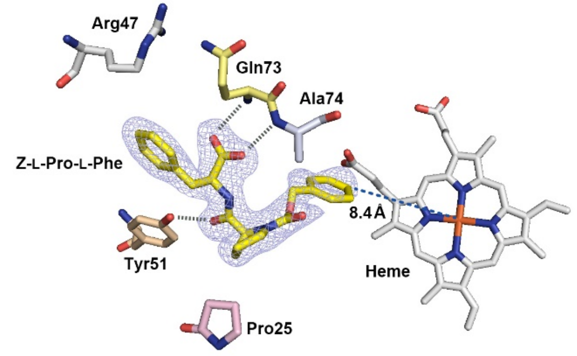


Figure 4. X-ray crystal structure of P450BM3 with Z-L-Pro-L-Phe (PDB code: 5XA3). $2F_o$-F_c electron-density map of Z-L-Pro-L-Phe contoured at the 1.0 σ level is shown in light-blue cross-hatching. The distance between the heme iron atom and the terminal carbon atom of Z-L-Pro-L-Phe is shown as a blue dashed line.

To confirm the binding of the decoy molecules, we attempted to crystallize P450BM3 with C9-L-Phe, *S*-Ibu-L-Phe, and Z-L-Pro-L-Phe and succeeded in determining the crystal structure of the Z-L-Pro-L-Phe-bound form of P450BM3 at 2.2 Å resolution (Fig. 4, PDB Code: 5XA3). In the fatty acid binding channel of P450BM3, a clear electron density corresponding to Z-L-Pro-L-Phe could be observed. While the structure of Z-L-Pro-L-Phe differs considerably from that of a basic fatty acid, it can be observed to bind in a similar fashion to PFC9-L-Trp through hydrogen-bonding interactions with three amino acids, Tyr51, Gln73, and Ala74 (Fig. 4). The distance between the terminal carbon atom of the phenyl

group of Z-L-Pro-L-Phe and the heme iron was 8.4 Å (Fig. 4). Docking simulations of benzene calculated using AutoDock Vina (Fig. S3) revealed that there is adequate space for benzene binding at the distal side of the heme prosthetic group, even after binding of Z-L-Pro-L-Phe. These observations are consistent with hydroxylation of benzene by P450BM3 in the presence of Z-L-Pro-L-Phe, while Z-L-Pro-L-Phe remains unhydroxylated (Fig. S4).

It is noteworthy that the proline side chain of Z-L-Pro-L-Phe occupied the space around Pro25 (Fig. 4). We presume that the bulkiness of proline at the center of Z-L-Pro-L-Phe may play a crucial role in fixing Z-L-Pro-L-Phe at the entrance of the fatty acid binding channel to create adequate space at the distal side of the heme for benzene binding. In fact, Z-Gly-L-Phe and Ph-C6-L-Phe did not efficiently activate P450BM3 for benzene hydroxylation, while *S*-Ibu-L-Phe, which possesses a bulky phenyl group at the center of its structure, promoted a catalytic activity that was many times higher. Simulated structures of *S*-Ibu-L-Phe bound to P450BM3 showed that the phenyl group including the methyl group next to the carbonyl moiety of ibuprofen may be located in proximity to Pro25 (Fig. S5a), similarly to the proline side chain of Z-L-Pro-L-Phe. Judging from the comparison between C10-L-Phe and Ph-C6-L-Phe, the terminal alkyl chain of C10-L-Phe may also be key for an efficient decoy molecule. Accordingly, we prepared *N*-heptyl-L-proline modified with L-phenylalanine (C7-L-Pro-L-Phe, Fig. 2) to further enhance catalytic activity. As expected, the turnover rate of C7-L-Pro-L-Phe reached 259 $min^{-1}P450^{-1}$ with a coupling efficiency of 43%. To our knowledge, this represents the highest turnover frequency (TOF) for benzene hydroxylation to phenol of any biocatalyst reported.[12] Docking simulations further supported the assumption that P450BM3 can accommodate C7-L-Pro-L-Phe in a similar fashion to Z-L-Pro-L-Phe (Fig. S5b). Finally, we estimated the TON for benzene hydroxylation using C7-L-Pro-L-Phe as a decoy molecule. The TON for a 12-h reaction using 25 nM P450BM3 reached 40,200 ± 1,700 with a coupling efficiency of 46%.

In conclusion, we have demonstrated that *N*-acyl amino acids as well as amino acid dipeptides strongly activate P450BM3 for benzene hydroxylation to generate phenol without formation of side products due to overoxidation of phenol, even though we did not perform any mutagenesis of P450BM3. We believe that the direct hydroxylation of benzene to phenol by P450BM3 assisted by the simple addition of amino acid derivatives as presented here could be an attractive alternative for phenol production. Despite having examined only a limited number of carboxylic acids and amino acids for the preparation of decoy molecules, further screening is expected to enhance the catalytic activity of P450BM3. We believe that the catalytic turnover rate and coupling efficiency for benzene hydroxylation can be improved further by optimizing the decoy molecule structure based upon the crystal structure of P450BM3 with Z-L-Pro-L-Phe, and in combination with mutagenesis to further tailor the active site for this reaction.[13]

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (S) to Y. W. (24225004) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan) and JST CREST Grant Number JPMJCR15P3, Japan. This work was also supported by JSPS KAKENHI Grant Number JP15H05806 in Precisely Designed Catalysts with Customized Scaffolding to O. S.

Keywords: benzene hydroxylation • cytochrome P450 • decoy molecule • *N*-acyl amino acids

Received: ((will be filled in by the editorial staff))
Published online on ((will be filled in by the editorial staff))

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