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Hydrogen Peroxide Dependent Monooxygenations by Tricking the Substrate Recognition of Cytochrome $P450_{BS\beta}$**

*Osami Shoji, Takashi Fujishiro, Hiroshi Nakajima, Misa Kim, Shingo Nagano, Yoshitsugu Shiro, and Yoshihito Watanabe**

The design and construction of enzymes as biocatalysts has been the subject of intensive studies because of their high regio- and enantioselectivities as well as high activities.[1,2] For example, site-directed mutagenesis and random mutagenesis are powerful tools to transform native enzymes into biocatalysts that are applicable to industrial processes. The construction of protein active sites by rational design is a good example of site-directed mutagenesis.[3] Although the mutation sites in the random mutagenesis (so-called "directed evolution") are rather unpredictable, effective substitution of amino acids to introduce desired functions has been demonstrated.[4,5] Cytochrome P450s (P450s), which are heme-containing monooxygenases, play important roles in drug metabolism, detoxification of xenobiotics, and steroid biosynthesis. In these reactions, P450s catalyze hydroxylation of less reactive C–H bonds, a key reaction in organic synthesis.[6,7] A series of P450 mutants have been prepared to alter their substrate specificity and improve catalytic activity.[8–10] Selective oxidation of saturated C–H bonds by biocatalysts is of great importance because of their potential use in industrial applications.[11,12] Alkane hydroxylation by engineered $P450_{cam}$[13] and P450 BM-3[8] has been reported, and Meinhold et al. and Xu et al. have recently prepared P450 mutants to catalyze ethane hydroxylation at relatively high turnover rates under mild conditions.[14,15] Unfortunately, the practical use of these P450s is limited, since they require the very expensive electron-donating cofactor NAD(P)H for the reductive oxygen activation. Although NAD(P)H-regeneration systems effectively reduce the cost, the catalytic systems become complicated and thus have other limitations.[16] Instead of the cofactors and molecular oxygen, P450s are capable of accepting peroxides as an oxidant; this is the so-called peroxide-shunt pathway. However, P450 systems using hydrogen peroxide (H_2O_2) are inefficient and impractical.[17,18]

In contrast to most P450s, $P450_{BS\beta}$ (CYP152A1), isolated from *Bacillus subtilis*, efficiently utilizes H_2O_2 to catalyze the exclusive hydroxylation of long-alkyl-chain fatty acids such as myristic acid to give β-hydroxymyristic acid (60%) and α-hydroxymyristic acid (40%).[19,20] This soluble enzyme does not require any cofactors, including electron-transfer systems; it works as a single component. Because of its low cost, H_2O_2 can be used as an oxidant in industrial-scale processes, and $P450_{BS\beta}$ would thus be a great candidate for practical biocatalysts. The X-ray crystal structure analysis of the palmitic acid bound form of $P450_{BS\beta}$[20] suggests a unique catalytic mechanism: 1) The catalytic reaction begins with the fixation of a substrate through interaction of the terminal carboxy group of the fatty acid with Arg^{242}, located near the heme; 2) A general acid–base function of the fatty acid–Arg^{242} salt bridge allows facile generation of the active species, the so-called Compound I,[21,22] to oxidize the substrate (Figure 1).[23] Without the interaction of the carboxy group, $P450_{BS\beta}$ does not accept H_2O_2 to start its reaction. Therefore, the fatty acid substrate itself is the initiator as well as the activator of the reaction.

As this unique catalytic mechanism contributes to high substrate specificity and regioselectivity of the hydroxylation,[19] $P450_{BS\beta}$ never oxidizes substrates other than fatty acids that have long alkyl chains. If one could trick $P450_{BS\beta}$ by using a decoy molecule with a carboxy group which is recognized by $P450_{BS\beta}$ as a substrate to make the carboxylate–Arg salt bridge but is not oxidized by $P450_{BS\beta}$, a wide variety of nonnatural substrates could be oxidized by the H_2O_2–$P450_{BS\beta}$ system (Scheme 1).

We report herein a unique approach to utilize $P450_{BS\beta}$ as a versatile monooxygenase by applying a simple substrate trick, namely, a series of short-alkyl-chain carboxylic acids.

The recombinant $P450_{BS\beta}$ with a 6× histidine tag was expressed in *Escherichia coli* and purified by nickel chelate affinity chromatography.[24] $P450_{BS\beta}$ showed no obvious catalytic activity toward hydroxylation of carboxylic acids with alkyl chains shorter than 10-carbon-atoms long. The alkyl tails of the short-alkyl-chain carboxylic acids would be too short to reach to the hydrophobic channel of $P450_{BS\beta}$, thus resulting in

[*] 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: yoshi@nucc.cc.nagoya-u.ac.jp
Dr. O. Shoji, T. Fujishiro, Dr. H. Nakajima
Department of Chemistry
Graduate School of Science
Nagoya University
Furo-cho, Chikusa-ku, Nagoya 464-8602 (Japan)
Dr. M. Kim, Dr. S. Nagano, Dr. Y. Shiro
RIKEN SPring-8 Center
Harima Institute
1-1-1 Kouto, Sayo, Hyogo 679–5148, (Japan)

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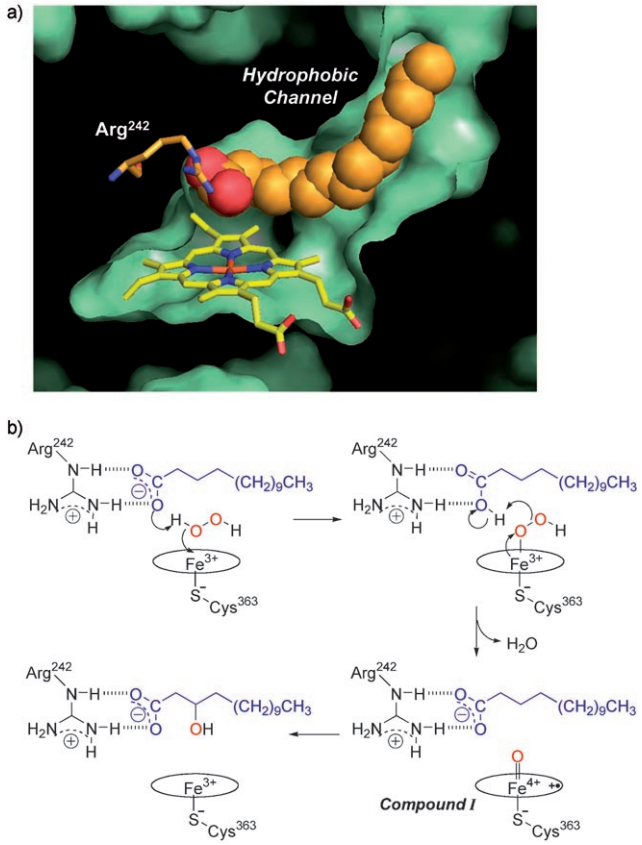
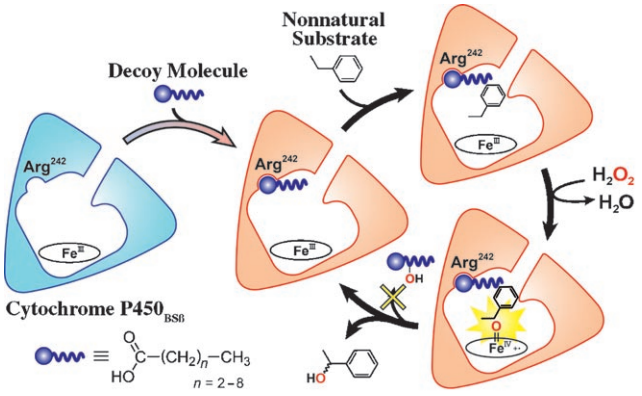


Figure 1. a) The active-site structure of $P450_{BS\beta}$ containing palmitic acid (PDB: 1IZO). The oxygen, nitrogen, and iron atoms are shown in red, blue, and brown, respectively. The heme and arginine 242 are shown as yellow and orange sticks, respectively. The bound palmitic acid is also shown as orange spheres. Hydrogen atoms are omitted for clarity. b) Proposed catalytic hydroxylation mechanism and the roles of the substrate carboxylate–Arg^{242} salt bridge.



Scheme 1. Our strategy for the oxidation of nonnatural substrates (e.g. ethylbenzene) in the presence of a decoy molecule. $P450_{BS\beta}$ is depicted as a triangle (P450s have a triangular-prism shape), and the decoy molecule is shown as a blue wavy line with a spherical head.

the loose fixation of the short alkyl chains and the failure of the hydroxylation (see the Supporting Information). This feature is similar to cytochrome $P450_{SP\alpha}$ (CYP152B1),[25] which shows 44% overall identity to $P450_{BS\beta}$.[19] The incomplete occupation of the hydrophobic channel by a shorter alkyl chain would allow another substrate access to the heme pocket (Scheme 1). In fact, one-electron oxidation of guaiacol was catalyzed by $P450_{BS\beta}$ at a high reaction rate in the presence of carboxylic acids with short alkyl chains, and heptanoic acid gave the maximum rate of 3750 turnovers min^{-1} protein^{-1} among carboxylic acids examined (Figure 2).

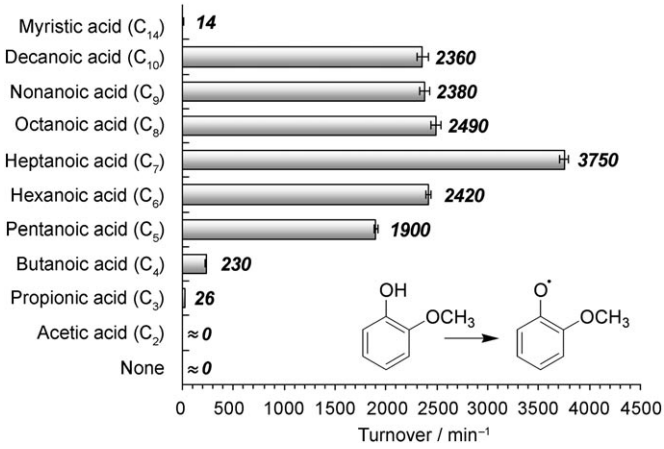
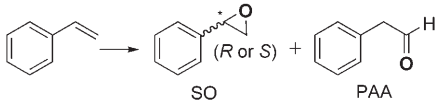


Figure 2. The effect of decoy molecules on the one-electron oxidation of guaiacol by $P450_{BS\beta}$. Shown are the initial turnover rates in the presence and in the absence of carboxylic acids. All values are the average of at least five measurements and expressed in units of (nmol product) min^{-1} (nmol P450)$^{-1}$. Error bars represent the standard deviation. The number in parentheses beside the name of carboxylic acid denotes the number of carbon atoms.

In contrast, no appreciable reaction was observed either in the absence of carboxylic acid or in the presence of myristic acid under the same conditions.

Epoxidation of styrene also proceeded at a maximum rate of 334 turnover min^{-1} with high enantioselectivity (80% *ee*) in the presence of hexanoic acid (Table 1). Furthermore, hydroxylation of the methylene carbon of ethylbenzene proceeded selectively and afforded the corresponding alcohol at

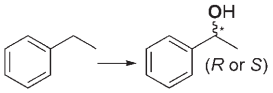
Table 1: Catalytic activity and enantioselectivity for styrene epoxidation by $P450_{BS\beta}$ in the presence of carboxylic acids.



Carboxylic acid	Rate [min^{-1}][a]	*ee* (*S*) [%]	SO:PAA[b]
butanoic acid (C_4)	136 ± 32	84 ± 2	87:13
pentanoic acid (C_5)	317 ± 14	84 ± 1	86:14
hexanoic acid (C_6)	334 ± 59	80 ± 1	80:20
heptanoic acid (C_7)	290 ± 40	83 ± 1	91:9
octanoic acid (C_8)	148 ± 28	85 ± 1	90:10

[a] The unit for catalytic activity is (nmol product) min^{-1} (nmol P450)$^{-1}$; uncertainty given as the standard deviation for five measurements. [b] SO: Styrene oxide, PAA: phenylacetaldehyde.

Table 2: Catalytic activity and enantioselectivity for ethylbenzene hydroxylation by $P450_{BS\beta}$ in the presence of carboxylic acids.



Carboxylic acid	Rate [min^{-1}][a]	*ee* (*R*) [%]
butanoic acid (C_4)	11 ± 1	35 ± 4
pentanoic acid (C_5)	20 ± 2	41 ± 4
hexanoic acid (C_6)	24 ± 2	51 ± 3
heptanoic acid (C_7)	28 ± 4	68 ± 2
octanoic acid (C_8)	10 ± 3	61 ± 3

[a] The unit for catalytic activity is (nmol product) min^{-1} (nmol P450)$^{-1}$; uncertainty given as the standard deviation for five measurements.

a rate of 28 turnover min^{-1} (68% *ee*) in the presence of heptanoic acid (Table 2). These results clearly indicate that $P450_{BS\beta}$ misrecognizes short-alkyl-chain carboxylic acids as the substrate owing to its structural similarity and catalyzes the oxidation of non-fatty acids. The rate of oxidation of myristic acid by $P450_{BS\beta}$ is reported in the range 159–365 turnover min^{-1},[20,24] whereas that of the guaiacol is 10fold higher. The results imply that the decoy molecule keeps the $P450_{BS\beta}$ catalytic cycle always "on", whereas the binding of a long alkyl chain in the active site turns the oxidation cycle "on". Furthermore, myristic acid was found to prohibit the nonnatural substrate oxidation, possibly because of the full occupation of the space of the heme vicinity by the alky chain.

The high enantioselectivity observed for the styrene epoxidation and the ethylbenzene hydroxylation suggests that the specific substrate binding site could be constructed by the combination of the protein scaffold and the decoy molecule. As the enantioselectivity of the ethylbenzene hydroxylation is extremely dependent on the structure of the decoy molecules, higher selectivity can be introduced by using chiral carboxylic acids. The initial turnover rates for monooxygenation by the H_2O_2–$P450_{BS\beta}$ system are even greater than those of chloroperoxidase (CPO), which is one of the most efficient hydrogen peroxide dependent biocatalysts. For example, wild-type CPO catalyzes the hydroxylation of ethylbenzene and the epoxidation of styrene at rates of 1.4 and 288 (nmol product) min^{-1} (nmol CPO)$^{-1}$, respectively.[26,27] That the catalytic activities are dependent on the alkyl-chain structure of the decoy molecule indicates that the catalytic activities may be further improved by the design of the decoy molecule.

As described herein, we have observed versatile H_2O_2-dependent monooxygenase activities including the hydroxylation of ethylbenzene into $P450_{BS\beta}$ without replacing any amino acid residues. To the best of our knowledge, this is the first example of the drastic change of substrate specificity without any mutagenesis. Furthermore, the efficient H_2O_2-dependent hydroxylation of nonnatural, saturated hydrocarbons catalyzed by P450 is a rare example. Although P450 BM-3 has been engineered to enhance the efficiency of the "hydrogen peroxide shunt" pathway by mutagenesis, it catalyzes the hydroxylation reaction of the natural substrate (fatty acid)[28,29] and the transformation of indole to indigo.[30]

By shortening the alkyl-chain length of the natural substrates, we have demonstrated a novel approach for the introduction of new functions and higher activities into $P450_{BS\beta}$ while keeping its intrinsic advantage, namely, the use of H_2O_2. By designing the nature and structure of the decoy molecule, $P450_{BS\beta}$ could be tailored to accommodate and oxidize a wide range of substrates. A combination of the decoy molecule with mutagenesis would enable us to create $P450_{BS\beta}$ systems that catalyze various hydroxylation reactions without consuming expensive cofactors, such as NAD(P)H.

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