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Chiral-Substrate-Assisted Stereoselective Epoxidation Catalyzed by H_2O_2-Dependent Cytochrome $P450_{SP\alpha}$

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Abstract: The stereoselective epoxidation of styrene was catalyzed by H_2O_2-dependent cytochrome $P450_{SP\alpha}$ in the presence of carboxylic acids as decoy molecules. The stereoselectivity of styrene oxide could be altered by the nature of the decoy molecules. In particular, the chirality at the α-positions of the decoy molecules induced a clear difference in the chirality of the product: (*R*)-ibuprofen enhanced the formation of (*S*)-styrene oxide, whereas (*S*)-ibuprofen preferentially afforded (*R*)-styrene oxide. The crystal structure of an (*R*)-ibuprofen-bound cytochrome $P450_{SP\alpha}$ (resolution 1.9 Å) revealed that the carboxylate group of (*R*)-ibuprofen served as an acid–base catalyst to initiate the epoxidation. A docking simulation of the binding of styrene in the active site of the (*R*)-ibuprofen-bound form suggested that the orientation of the vinyl group of styrene in the active site agreed with the formation of (*S*)-styrene oxide.

Keywords: carboxylic acids · chirality · decoy molecules · epoxidation · hydrogen peroxide

Introduction

Oxidation reactions catalyzed by biocatalysts have been of much interest in the field of organic synthesis because these biocatalysts are able to produce fine chemicals, including chiral building blocks and pharmaceuticals, under mild conditions.[1] Cytochrome P450s (P450s) are ubiquitous enzymes that are comprised of a superfamily of heme-containing monooxygenases and are involved in oxidative metabolism, detoxification, and in the synthesis of steroids.[2] P450s have been regarded as attractive candidates as oxidation catalysts because of their high catalytic activity for the direct insertion of oxygen into unactivated C–H bonds.[3] However, P450s consume a stoichiometric amount of an expensive cofactor (NAD(P)H) in the reductive activation of molecular oxygen.[3,4] Thus, the use of P450s in organic synthesis has been limited to the formation of valuable products, such as fine chemicals and drugs.[4] To overcome this limitation, Shimizu and co-workers developed a hydrogen-peroxide-dependent $P450_{BM3}$ by site-directed mutagenesis.[5] Whilst wild-type $P450_{BM3}$ did not efficiently use hydrogen peroxide for the generation of the active species (Compound I) followed by the hydroxylation of fatty acids,[6] the F87A mutant of $P450_{BM3}$ accepted hydrogen peroxide and catalyzed hydroxylation reactions without the consumption of NADPH. Although this mutant has been further developed to improve its catalytic activity by directed evolution,[7] the final mutant still requires a relatively high concentration of hydrogen peroxide.

In contrast to most P450s, including $P450_{BM3}$, hydrogen-peroxide-dependent P450s, such as $P450_{BS\beta}$,[8] $P450_{SP\alpha}$,[9] and $P450_{CLA}$,[10] exclusively use hydrogen peroxide as an oxidant and efficiently catalyze the site-specific hydroxylation of fatty acids. Although these P450s are expected to be practical biocatalysts, these enzymes usually show very high substrate specificity. The crystal structure of a palmitic-acid-bound form of $P450_{BS\beta}$ revealed that the carboxylate of palmitic acid interacted with the Arg-242 moiety that was located near the heme.[11] The location of the carboxylate group was almost the same as that of the distal glutamate in chloroperoxidase (CPO), which is one of the most efficient hydrogen-peroxide-dependent biocatalysts.[12] This observation supported a proposal that the general acid–base function for the facile generation of the active species was provided by the carboxy group of the fatty acid that was bound to $P450_{BS\beta}$ (Scheme 1 a).[13] This unique reaction mechanism of $P450_{BS\beta}$ also contributed to its high substrate specificity and $P450_{BS\beta}$ did not oxidize substrates other than long-alkyl-chain fatty acids. However, we have demonstrated that the oxidation reactions of non-natural substrates other than long-alkyl-chain fatty acids could be catalyzed by $P450_{BS\beta}$ in the presence of a series of short-alkyl-chain carboxylic acids as "decoy molecules" that induced the substrate-misrecognition of $P450_{BS\beta}$.[14] The addition of decoy molecules allowed

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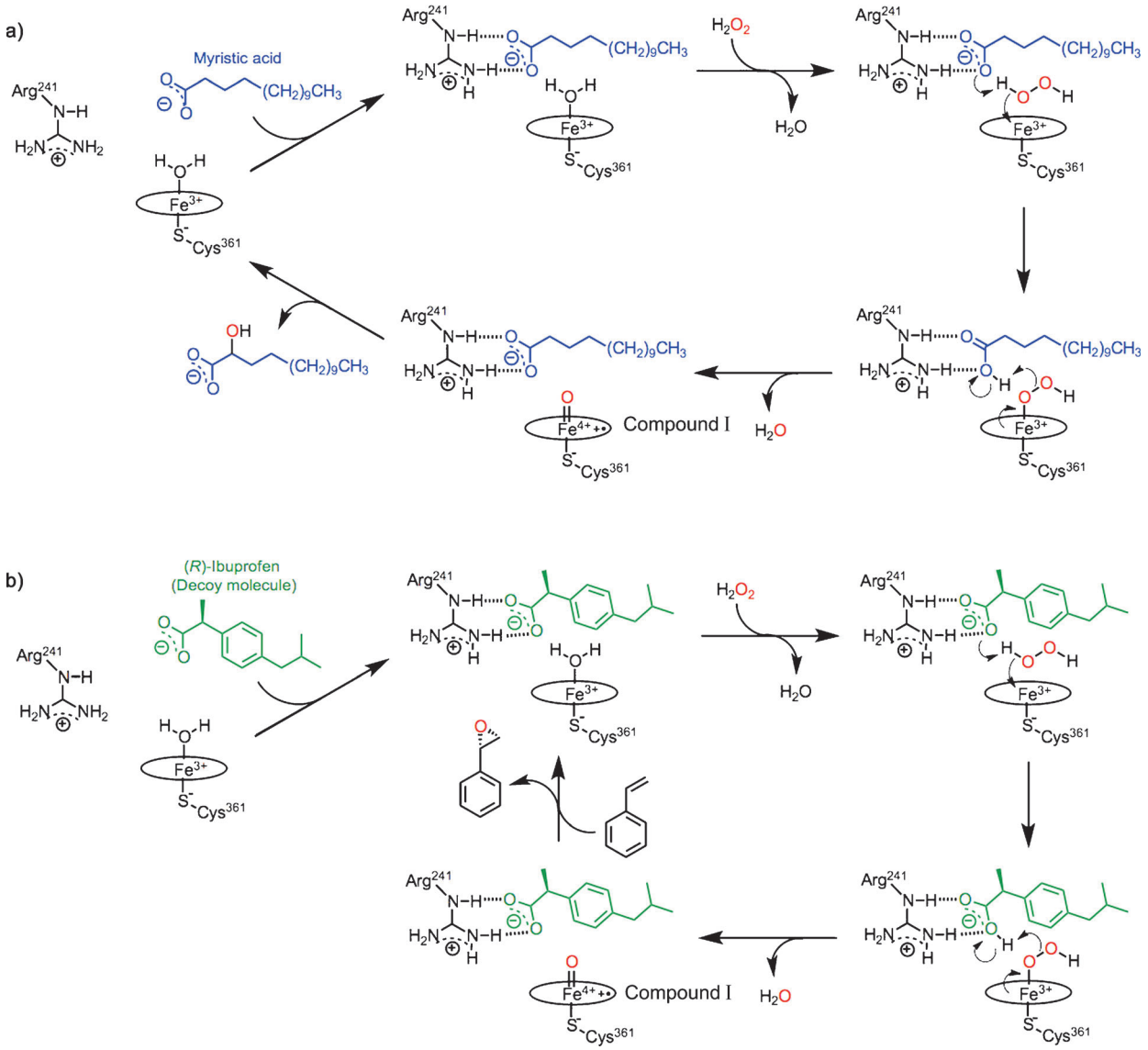

Scheme 1. a) Proposed reaction mechanism for the hydroxylation of long-alkyl-chain fatty acids catalyzed by $P450_{SP\alpha}$. The carboxylate group of the fatty acid served as a general acid–base catalyst to generate Compound I species by using H_2O_2 as an oxidant. b) Possible reaction mechanism for the oxidation of non-natural substrates (styrene) other than long-alkyl-chain fatty acids catalyzed by $P450_{SP\alpha}$ in the presence of a carboxylic acid as a "decoy molecule" ((*R*)-ibuprofen, green) that induced the substrate-misrecognition of $P450_{SP\alpha}$.

Abstract in Japanese:

長鎖脂肪酸を選択的に水酸化する過酸化水素駆動型シトクロム $P450_{SP\alpha}$ に、カルボキシル基を有する基質類似分子（デコイ分子）を添加するとスチレンのエポキシ化反応が進行し、デコイ分子のキラリティにより生成物のエナンチオ選択性が大きく変化することを見出した。(*R*)-イブプロフェンとシトクロム $P450_{SP\alpha}$ の共結晶化と結晶構造解析に成功し、(*R*)-イブプロフェンが結合することで形成される反応空間に、スチレンが *S* 配置のスチレンオキシドを与える配向で取り込まれることを明らかにした。

$P450_{BS\beta}$ to generate the active species and catalyze a variety of reactions, such as the oxidations of guaiacol, styrene, ethylbenzene, 1-methoxynaphthalene, and thioanisole.[14–15] Thus, the use of decoy molecules is a useful method for the generation of active species and for the alteration of substrate specificity. In these "substrate-misrecognition systems," the carboxylate group of the decoy molecule serves as a general acid–base catalyst. X-ray crystal-structure analysis of a heptanoic-acid (decoy molecule)-bound form of $P450_{BS\beta}$ showed electron density that corresponded to the carboxylate of heptanoic acid at the same position as that of palmitic acid.[13] More recently, we solved the X-ray structure of the palmitic-acid-bound form of $P450_{SP\alpha}$ (Figure 1),[16] which had 44% amino-acid-sequence identity to $P450_{BS\beta}$ and also catalyzed the hydroxylation of fatty

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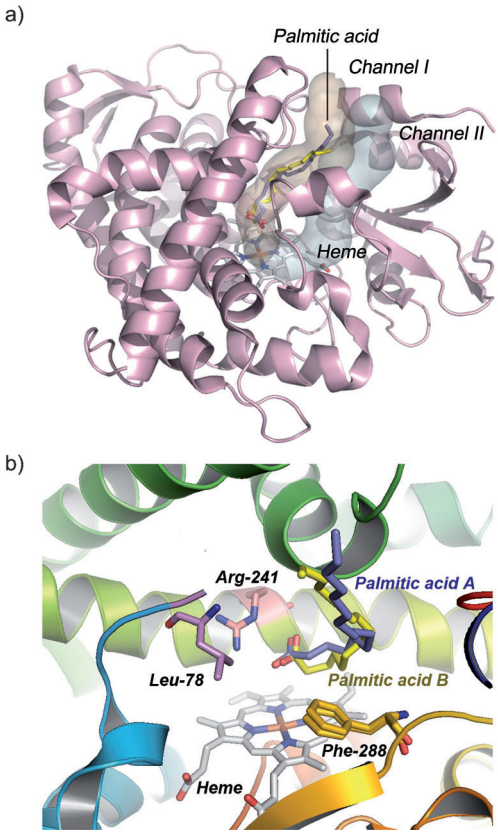


Figure 1. a) Overall structure of H_2O_2-dependent cytochrome $P450_{SP\alpha}$ with palmitic acid:[16] The two channels were represented as light-brown (Channel I) and light-blue surfaces (Channel II). b) Structure of the active site around the heme group. Heme, Arg-241, Leu-78, and Phe-288 are represented as stick models. Palmitic acids of Conformation A (blue) and Conformation B (yellow, the productive conformation that afforded the α-hydroxy fatty acid) are shown as stick models.

acids. The regioselectivity of $P450_{SP\alpha}$ was 100% α-selective, whilst $P450_{BS\beta}$ hydroxylated both the α- and β positions with a ratio of 43:57.[8–9]

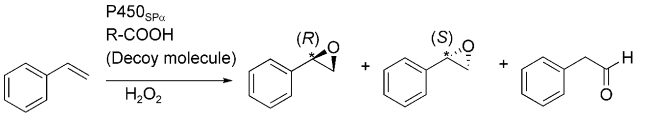
The crystal structure of the palmitic-acid-bound form of $P450_{SP\alpha}$ revealed that the key interactions between the carboxylate group of palmitic acid and the guanidine group of the arginine moiety near the heme site were conserved in $P450_{SP\alpha}$. Although two alternative conformations of palmitic acid were observed (Figure 1b), the distance between the carboxylate oxygen atom and the heme iron was 5.2 Å for Conformation A and 5.5 Å for Conformation B (a productive conformation that gives the α-hydroxy fatty acid), thus indicating that the location of the oxygen atom was similar to that of the terminal carboxylate group of palmitic acid in $P450_{BS\beta}$ (5.3 Å). These observations indicated that the reaction mechanism for the formation of compound I of $P450_{SP\alpha}$ was the same as that of $P450_{BS\beta}$ (Scheme 1a). Encouraged by the crystallographic studies of $P450_{SP\alpha}$, we decided to explore whether the monooxygenation system with decoy molecules could be expanded to $P450_{SP\alpha}$ (Scheme 1b). We expected that $P450_{SP\alpha}$ would show a different stereoselectivity to that of $P450_{BS\beta}$ because $P450_{SP\alpha}$ catalyzes the hydroxylation of fatty acids with high regioselectivity (α-selective) and stereoselectivity (*S*-selective).[16] We were interested in how the catalytic properties of the oxidation of unnatural substrates were affected by the structure of the active site of $P450_{SP\alpha}$, as well as the structure of decoy molecules. Herein, we report the stereoselective oxidation of styrene catalyzed by $P450_{SP\alpha}$ in the presence of a variety of decoy molecules, including chiral decoy molecules such as (*R*)- and (*S*)-ibuprofen. In addition, we performed the crystal-structure analysis of (*R*)-ibuprofen-bound $P450_{SP\alpha}$ in which electron density that corresponded to (*R*)-ibuprofen was observed. The crystal structure of the (*R*)-ibuprofen-bound form allowed us to discuss the effect of the chirality of (*R*)-ibuprofen on its binding and the stereoselectivity of the styrene-epoxidation reaction.

Results and Discussion

Styrene Oxidation in the Presence of Carboxylic Acids

Initially, we investigated whether $P450_{SP\alpha}$ catalyzed the epoxidation of styrene in the presence of a series of short-alkyl-chain carboxylic acids as decoy molecules. The styrene-epoxidation reaction was catalyzed in the presence of a decoy molecule to give the corresponding styrene oxide. The turnover numbers were heavily dependent on the alkyl-chain length of the carboxylic acids and were up to 165 min^{-1} in the presence of heptanoic acid for wild-type $P450_{SP\alpha}$ (Table 1). This result clearly showed that the carboxylate group of short-alkyl-chain carboxylic acids served as an acid–base catalyst for the efficient generation of active species, as was observed for $P450_{BS\beta}$, thereby indicating that

Table 1. Epoxidation of styrene catalyzed by WT, L78F, and F288G $P450_{SP\alpha}$ in the presence of carboxylic acids.



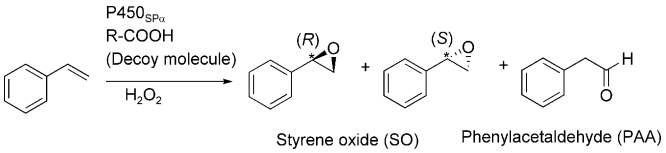
Styrene oxide (SO) Phenylacetaldehyde (PAA)

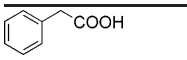
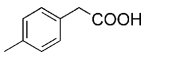
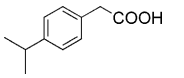
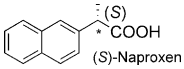
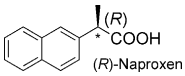
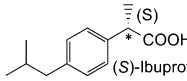
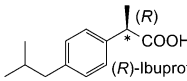
Decoy molecule (carbon-chain length)	$P450_{SP\alpha}$	Rate [min^{-1}][a]	*ee* [%] (*R*/*S*)	SO/PAA
octanoic acid (C8)	WT	96(±8)	22(±1) (*R*)	76:24
	L78F	164(±18)	30(±1) (*R*)	73:27
	F288G	124(±17)	38(±2) (*S*)	66:34
heptanoic acid (C7)	WT	165(±11)	25(±2) (*R*)	76:24
	L78F	277(±32)	34(±1) (*R*)	72:27
	F288G	180(±59)	34(±4) (*S*)	66:34
hexanoic acid (C6)	WT	139(±16)	28(±1) (*R*)	76:24
	L78F	502(±37)	32(±1) (*R*)	74:26
	F288G	228(±10)	27(±3) (*S*)	63:37
pentanoic acid (C5)	WT	145(±8)	36(±6) (*R*)	76:24
	L78F	319(±74)	31(±1) (*R*)	73:27
	F288G	322(±5)	18(±3) (*S*)	67:33
butyric acid (C4)	WT	106(±6)	33(±6) (*R*)	75:25
	L78F	310(±26)	34(±1) (*R*)	73:27
	F288G	354(±28)	16(±1) (*S*)	69:31

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

this monooxygenation system with decoy molecules was not only applicable to $P450_{BS\beta}$ but also to $P450_{SP\alpha}$. Interestingly, in contrast to $P450_{BS\beta}$, which gave (*S*)-styrene oxide, $P450_{SP\alpha}$ preferentially gave (*R*)-styrene oxide in the presence of heptanoic acid under the same conditions. Structural comparison of the active site of $P450_{SP\alpha}$ with that of $P450_{BS\beta}$ showed that Phe-288 and Leu-78 moieties in $P450_{SP\alpha}$[16] were replaced by Gly-290 and Phe-79 in $P450_{BS\beta}$, respectively.[11] These amino acids appeared to be key residues in determining the stereoselectivity; therefore, we examined the stereoselectivity of the styrene-epoxidation reactions catalyzed by L78F and F288G mutants. Whereas the stereoselectivity of the L78F mutant was unchanged, the F288G mutant gave the opposite stereoselectivity to that catalyzed by WT $P450_{SP\alpha}$. The mutation of Phe-288 into Gly-288 in $P450_{SP\alpha}$ might have provided a similar active site of $P450_{BS\beta}$ and, hence, gave (*S*)-styrene oxide. Interestingly, the L78F and F288G mutants gave higher catalytic activity than WT. In particular, the turnover of the L78F mutant with hexanoic acid was more than 500 min^{-1}, which is, to the best of our knowledge, the highest catalytic activity for the epoxidation of styrene among the heme enzymes with hydrogen peroxide reported so far.[7,17] To examine the effect of decoy molecules on the catalytic properties, we carried out the epoxidation of styrene with a variety of decoy molecules that contained an aromatic ring because the aromatic ring of the decoy molecule was expected to interact with both Phe-288 and with the aromatic group of styrene. The decoy molecules used herein, as well as the results of the styrene-epoxidation reactions, are summarized in Table 2. The stereoselectivity was drastically altered in the range 39% *ee* (*R*) to 88% *ee* (*S*) by changing the combination of the decoy molecule and the mutant. Among the decoy molecules examined, (*R*)-ibuprofen afforded the highest enantioselectivity in the case of WT $P450_{SP\alpha}$ and the combination of (*R*)-ibuprofen and the F288G mutant gave 88% *ee* (*S*). In addition, the chirality of ibuprofen induced a clear difference: (*R*)-ibuprofen gave (*S*)-styrene oxide, whilst (*S*)-ibuprofen gave (*R*)-styrene oxide with WT $P450_{SP\alpha}$, thus showing that (*R*)-ibuprofen was effective in enhancing *S*-selectivity. The *R*-chirality at the α-carbon atoms of the decoy molecules seemed to be important for enhancing *S*-selectivity. Indeed, (*R*)-naproxen also enhanced *S*-selectivity. These results showed that the stereoselectivity could be controlled by simply selecting the *R*- or *S*-enantiomer of ibuprofen.

Table 2. Epoxidation of styrene catalyzed by WT and F288G $P450_{SP\&\#945;}$ in the presence of carboxylic acids that contained an aromatic ring.



Decoy molecule	$P450_{SP\alpha}$	Rate [min^{-1}][a]	*ee* [%] (*R*/*S*)	SO/PAA
	WT	189(±30)	39(±2) (*R*)	72:28
	F288G	89(±15)	22(±1) (*S*)	74:26
	WT	217(±25)	27(±1) (*R*)	77:23
	F288G	180(±32)	29(±1) (*S*)	72:28
	WT	302(±31)	11(±3) (*R*)	77:23
	F288G	281(±36)	50(±1 (*S*)	74:26
 (*S*)-Naproxen	WT	94(±9)	26(±1) (*R*)	76:24
	F288G	90(±15)	22(±1) (*S*)	68:32
 (*R*)-Naproxen	WT	160(±5)	23(±1) (*S*)	83:17
	F288G	151(±20)	37(±1) (*S*)	69:31
 (*S*)-Ibuprofen	WT	43(±4)	4(±1) (*R*)	82:18
	F288G	12(±1)	31(±2) (*S*)	73:27
 (*R*)-Ibuprofen	WT	92(±11)	63(±3) (*S*)	95:05
	F288G	176(±27)	88(±1) (*S*)	90:10

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

Crystal-Structure Analysis

To understand the asymmetric epoxidation reaction catalyzed by $P450_{SP\alpha}$ with ibuprofen, in particular the effect of the chirality of the α-carbon atom of ibuprofen on the chirality of the products, we determined the crystal structure of an (*R*)-ibuprofen-bound form of wild-type $P450_{SP\alpha}$ at a resolution of 1.9 Å (Figure 2), which clearly showed the electron density of (*R*)-ibuprofen in the active site. No remarkable structural differences were observed between the overall structures of the palmitic-acid-bound form and the (*R*)-ibuprofen-bound form of wild-type $P450_{SP\alpha}$. Although the carboxylate was slightly separated from the heme moiety (0.7 Å, Figure 2c), the location of the carboxylate group of (*R*)-ibuprofen was essentially the same as that of palmitic acid. The distance between one of the carboxylate oxygen atoms of (*R*)-ibuprofen and the heme iron atom was 5.2 Å. Thus, the carboxylate group of (*R*)-ibuprofen was expected to serve as an acid–base catalyst for the generation of active species with hydrogen peroxide. The phenyl ring of (*R*)-ibuprofen interacted with the phenyl rings of Phe-287 and Phe-288 in an edge-to-face manner. The *para*-isobutyl group of (*R*)-ibuprofen was held close to the side-chain of Leu-77 through hydrophobic interactions. The methyl group at the α-position of (*R*)-ibuprofen protruded toward the heme moiety. Even after the binding of (*R*)-ibuprofen, there was space to accommodate a styrene molecule. The channel that was used for the access of fatty acids (Channel I) was occupied by (*R*)-ibuprofen (Figure 1a), but Channel II was still accessible for additional foreign substrates. Styrene may have accessed the heme cavity through Channel II.

Although we wanted to examine the differences between the active-site structures upon binding (*R*)- and (*S*)-ibuprofen, we could not obtain crystals of $P450_{SP\alpha}$ with (*S*)-ibuprofen. The crystal structure of the (*R*)-ibuprofen-bound form showed that the distance between the α-carbon atom and the side-chain methyl group of Ala-245 was 3.5 Å (Figure 2b). This result implied that, if (*S*)-ibuprofen tended to stay in the same location as (*R*)-ibuprofen, the methyl group

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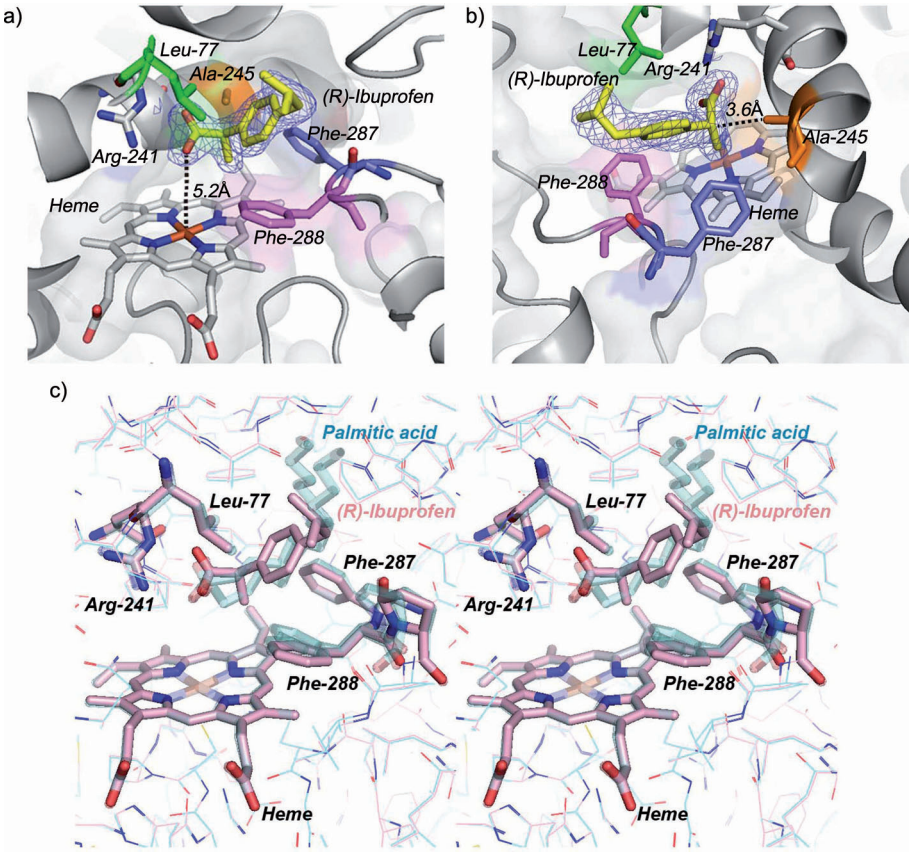

Figure 2. X-ray crystal structure of the (*R*)-ibuprofen-bound form of $P450_{SP\alpha}$ (PDB code: 3VM4). Views from the propionate side of the heme (a) and from the opposite side (b). The distance between the heme iron atom and the oxygen atoms of the carboxy group, as well as between the α-carbon and the methyl group in the side-chain of Ala-245, are shown with dotted lines. c) $2F_o$-F_c electron-density map of (*R*)-ibuprofen, contoured at the 1.0σ level (blue mesh). The hydrophobic amino-acid residues, heme, Arg-241, and (*R*)-ibuprofen are represented as stick models. Stereo view of the co-crystal structure with (*R*)-ibuprofen (pink) superimposed on the palmitic-acid-bound form of $P450_{SP\alpha}$ (PDB code: 3AWM, light blue).

of (*S*)-ibuprofen at the α-carbon atom would induce a steric repulsion with the side-chain methyl group of Ala-245. This steric repulsion may have led to a higher dissociation constant of (*S*)-ibuprofen, whereas we could not estimate the dissociation constants of (*R*)- and (*S*)-ibuprofen owing to no appreciable change in the UV/Vis spectra of $P450_{SP\alpha}$ upon the addition of ibuprofen, as was observed for the binding of fatty acids.[16] Whilst the possible location of (*S*)-ibuprofen is still unclear, it would be different from that of (*R*)-ibuprofen.

Docking Simulations

To understand the formation of the (*S*)-epoxide in the presence of (*R*)-ibuprofen, the binding mode of styrene in the active site of the (*R*)-ibuprofen-bound form was simulated by using Autodock4 (Figure 3).[18] In the simulated structure, the vinyl group of styrene was close to the heme iron (4.7 Å). The phenyl ring of styrene interacted with the phenyl rings of Phe-288. The orientation of the vinyl group of styrene in the active site agreed with the formation of (*S*)-styrene oxide. Because (*R*)-styrene oxide was obtained in the presence of pentanoic acid (36% *ee*) as a decoy molecule, the binding of styrene toward the pentanoic acid-bound form of $P450_{SP\alpha}$ was also simulated for comparison. We assumed that pentanoic acid would be accommodated at the active site of $P450_{SP\alpha}$ with the same fashion of palmitic acid. The structure of the pentatonic-acid-bound form was generated by simply shortening the alkyl chain of palmitic acid in the palmitic-acid-bound form of $P450_{SP\alpha}$. Because the crystal structure of $P450_{SP\alpha}$ with palmitic acid showed two alternative conformations of palmitic acid (Conformation A and Conformation B), the docking simulations were carried out toward both conformations. In the simulated structures, the vinyl groups of styrene-A and styrene-B were slightly further from the heme iron atom (Figure 3b) compared with that in the active site of the (*R*)-ibuprofen-bound form, but the orientation agreed with formation of (*R*)-styrene oxide. Because styrene interacted with the phenyl ring of Phe-288 and the side-chain of Gln-84 rather than (*R*)-ibuprofen and pentanoic acid (Figure 4), the orientation of styrene appeared to be governed by the conformation of Phe-288. This assumption was supported by the fact that the stereoselectivity was heavily affected by the mutation at Phe-288: the replacement of Phe-288 by glycine largely enhanced the formation of (*S*)-styrene oxide (Table 1 and 2).

Conclusions

We have demonstrated the stereoselective epoxidation of styrene catalyzed by $P450_{SP\alpha}$ with carboxylic acids as decoy molecules and confirmed that this monooxygenation system[14–15] was applicable to $P450_{SP\alpha}$. The crystal structure of $P450_{SP\alpha}$ with (*R*)-ibuprofen revealed that the carboxylate group of (*R*)-ibuprofen served as an acid–base catalyst to initiate the epoxidation. The stereoselectivity of the styrene-epoxidation reaction was largely affected by the chirality of ibuprofen and (*R*)-ibuprofen enhanced the formation of (*S*)-styrene oxide. We believe that our strategy with decoy mole-

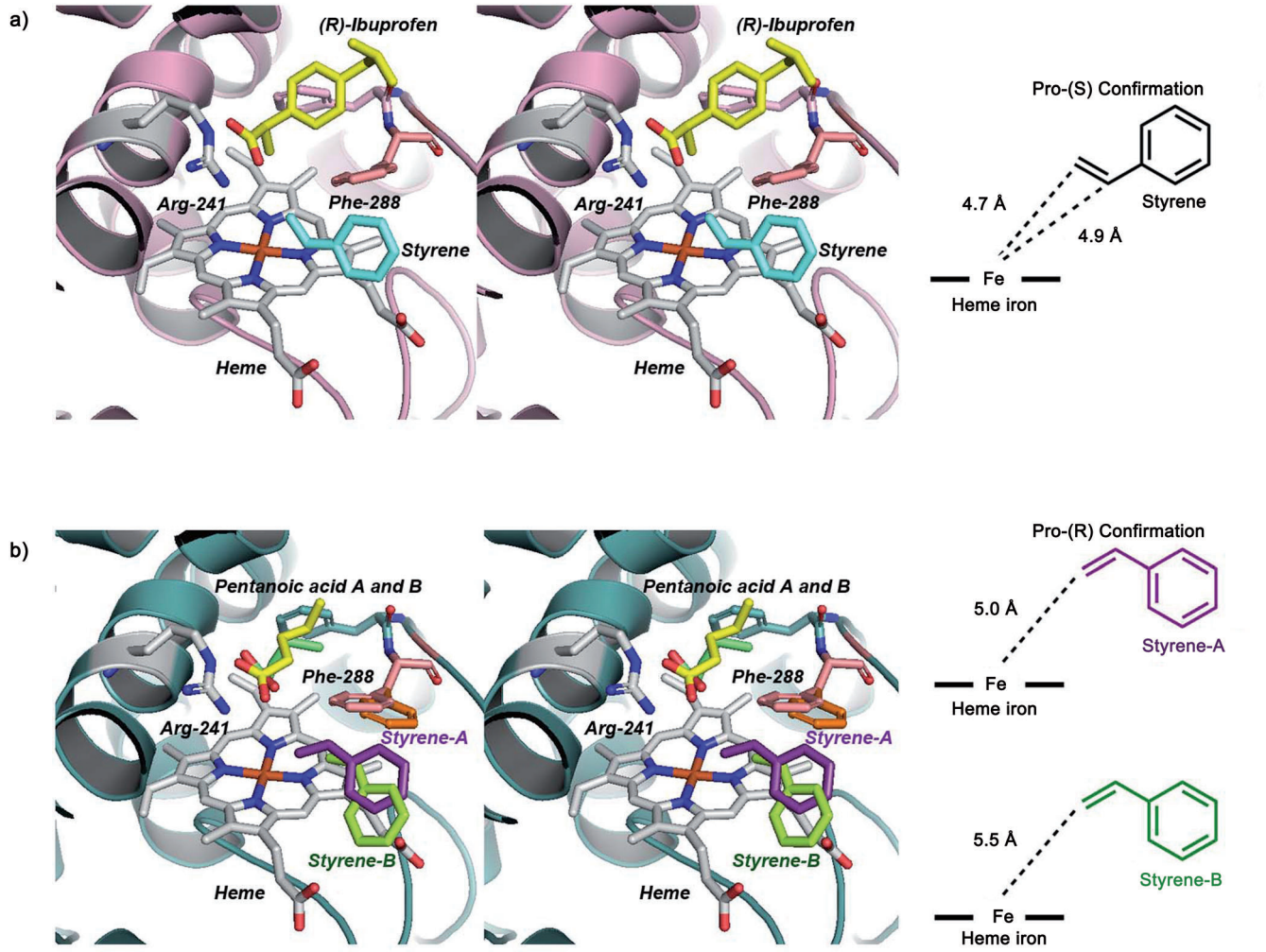

Figure 3. Docking simulations of styrene toward $P450_{SP\alpha}$ with (*R*)-ibuprofen (a) and pentatonic acid (b); the structure of $P450_{SP\alpha}$ with pentatonic acid that was used for docking simulations was generated from the crystal structure of $P450_{SP\alpha}$ with palmitic acid (Conformations A and B). Styrene-A (purple) and styrene-B (green) correspond to the results of docking simulations toward $P450_{SP\alpha}$ with pentanoic acid of Conformation A and Conformation B, respectively. The location of styrene is shown on the right.

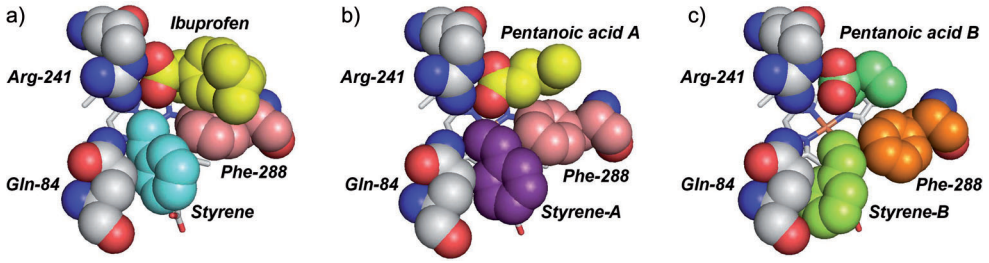

Figure 4. Docking simulations of styrene toward $P450_{SP\alpha}$ with (*R*)-ibuprofen (a) and pentatonic acid (b, c). Styrene-A (purple) and styrene-B (light green) correspond to the results of docking simulations toward $P450_{SP\alpha}$ with pentanoic acid of Conformation A and Conformation B, respectively.

cules can be applied to most of the hydrogen-peroxide-dependent P450s having the substrate-assisted reaction mechanism.[19] Furthermore, the combination of the decoy molecule and a mutant (or other P450s) enabled us to improve the catalytic activity, as well as the stereoselectivity of the products.

Experimental Section

Materials

All reagents and solvents were purchased from commercial sources and used without further purification, except for styrene. *n*-Butyric acid, hexanoic acid, octanoic acid, CH_2Cl_2, phosphoric acid, glycerol, KCl, HCl, and di-potassium hydrogen phosphate were purchased from Nacalai

Tesque Inc. (Kyoto, Japan). Heptanoic acid, 1,2-epoxyethylbenzene, (*R*)-(+)-1,2-epoxyethylbenzene, (*p*-isopropylphenyl)acetic acid, (*S*)-(+)-naproxen, and hydrogen peroxide were obtained from WAKO Pure Chemical Industries, Ltd (Osaka, Japan). Styrene, methyl phenylacetate, *n*-valeric acid, and (*S*)-(+)-ibuprofen were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). *p*-Methylphenylacetic acid, (*R*)-(−)-naproxen, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and (±)-2-methyl-2,4-pentanediol (MPD) were purchased from Sigma–Aldrich Co. (USA). (*R*)-(−)-Ibuprofen was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). 2-(*N*-morpholino)ethanesulfonic acid (MES) was purchased from Dojindo Laboratories (Kumamoto, Japan). Phenylacetic acid was prepared by the hydrolysis of methyl phenylacetate and purified by re-crystallization.

Measurements

UV/Vis spectra were recorded on a Shimadzu UV-2400 PC spectrophotometer. GC analysis was performed on a Shimadzu GC-2014 that was equipped with a cyclosil-β column (Agilent Technologies, Inc., 30 m × 0.25 mm).

Preparation of WT, L78F, and F288G P450$_{SP\alpha}$

WT, L78F, and F288G P450$_{SP\alpha}$ were prepared according to literature procedures.[16] The concentrations of WT P450$_{SP\alpha}$ and the mutants were determined from their CO difference spectra.[20]

Epoxidation of Styrene

Styrene was purified by column chromatography on alumina to remove the polymerization inhibitor before measurements; styrene was used immediately on purification.[21] The epoxidation reactions were performed as follows: 4 mM styrene, 4 mM H_2O_2, and 1 μM WT P450$_{SP\alpha}$ were mixed in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. A solution of the carboxylic acid in EtOH was added as a decoy molecule to a final concentration of 20 mM, except for (*R*)- and (*S*)-naproxen; because of their poor solubility, the concentrations of (*R*)- and (*S*)-naproxen were 5 mM in the reaction mixture. The reactions were performed at least three times with each decoy molecule. CH_2Cl_2 was added immediately into the reaction mixture for quenching and methyl phenylacetate was added as an internal standard. The extract was evaporated and the resulting solution was analyzed by GC that was equipped with a cyclosil-β column (Agilent Technologies, Inc.). The absolute configuration of styrene oxide was determined by using an authentic sample of (*R*)-styrene oxide. GC analytical conditions were as follows: injector temperature: 200 °C, detector temperature: 250 °C, initial oven temperature: 90 °C (30 min), ramp rate: 10 °C min^{-1}, final oven temperature 190 °C (20 min), carrier gas: He.

Co-Crystallization of (R)-Ibuprofen-Bound P450$_{SP\alpha}$ WT

P450$_{SP\alpha}$ WT that is expressed in *E. coli* contains palmitic acid, even after purification.[16] After the removal of glycerol and KCl from P450$_{SP\alpha}$ by solvent exchange to 0.1 M KPi buffer (pH 7.0), residual fatty acids, including palmitic acid, were removed from P450$_{SP\alpha}$ by passing thorough a Hydroxyalkoxypropyl-Dextran, Type VI column (Sigma–Aldrich, Co., USA) that was equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) at RT. The fractions were collected and the solvent was exchanged to 50 mM MES buffer that contained 20% glycerol (pH 7.0). The resulting P450$_{SP\alpha}$ was used as its substrate-free form. Co-crystallization of substrate-free P450$_{SP\alpha}$ WT and (*R*)-ibuprofen were performed by a sitting-drop vapor-diffusion method. The reservoir solution was prepared by mixing 0.1 M HEPES-NaOH (99 μL, pH 7.0), 35% MPD, and 1 M (*R*)-ibuprofen (1 μL) in EtOH to prepare a total solution volume of 100 μL. A solution of substrate-free P450$_{SP\alpha}$ WT (2 μL) was mixed with the reservoir solution (2 μL). Crystals were grown at 20 °C over 1 week.

Data Collection and Refinement

Crystals were flash-cooled in liquid nitrogen. X-ray-diffraction data sets were collected on beam-line BL26B1 that was equipped with an ADSC Quantum 315 CCD detector at the RIKEN SPring-8 (Hyogo, Japan) with a 1.0 Å wavelength at 100 K. The program HKL2000[22] was used to integrate the diffraction intensities and scaling. The structure of (*R*)-ibuprofen-bound P450$_{SP\alpha}$ was solved by molecular replacement with MolRep.[23] Model building and refinement were performed by using COOT[24] and REFMAC5.[25] The (*R*)-ibuprofen model was generated by using a Dundee PRODRG server[26] and used in the refinement with COOT and REFMAC5. Alternative conformations were introduced to Glu-89 with occupancies of 0.6 and 0.4, Arg-187, Val-313, and Ser-344 with occupancies of 0.7 and 0.3, and to Glu-64, Val-292, and His-412 with occupancies of 0.5 and 0.5. TLS refinement[27] was performed at the final stage of the refinement by defining each chain in the asymmetric unit as a separate TLS group. The produced model showed a final R_{fact} = 15.8% and R_{free} = 18.6% (Table 3). The final model consisted of one polypeptide chain with residues 9–415 of P450$_{SP\alpha}$, 1 heme, 1 (*R*)-ibuprofen, 2 (*R*)-2-methyl-2,4-pentanediol (MRD), and 274 water molecules. Structure validation was performed by using PROCHECK.[28] All protein figures were depicted by using PyMOL.[29]

Docking Simulations

Table 3. Data collection and refinement of (*R*)-ibuprofen-bound WT P450$_{SPa}$.

Data collection	
λ [Å]	1000
space group	$P3_121$
cell dimensions	
a, b, c [Å]	94.390, 94.390, 112.863
α, β, γ [°]	90.000, 90.000, 120.000
resolution [Å]	20.0–1.94 (2.01-1.94)
no. of total observed reflections	452029
no. of unique reflections	43560
R_{merge}[a,b] [%]	4.8 (29.7)
completeness[a] [%]	100 (100)
$I/\sigma(I)$[a]	53.1 (8.2)
redundancy[a]	10.4 (10.7)
Refinement statistics	
resolution range [Å]	19.76–1.94
no. of monomer/asymmetric unit	1
R_{fact}/R_{free}[c,d] [%]	15.8/18.6
r.m.s.d. bond length[e] [Å]	0.012
r.m.s.d. bond angles[e] [°]	1.193
no. of atoms	3614
average *B*-factor [Å^2]	19.5

[a] The values in parentheses are for the highest-resolution shell. [b] $R_{merge} = \Sigma|I - <I>|/\Sigma I$. [c] $R_{fact} = \Sigma||F_o| - k|F_c||/\Sigma|F_o|$ where F_o and F_c are the observed and calculated structure-factor amplitudes, respectively. [d] R_{free} was calculated as the R_{fact} for 5% of the reflection that were not included in the refinement. [e] r.m.s.d. = root mean square deviation.

The docking experiments were performed by using AutoDock4[18] and AutoGrid4 in combination with AutoDock Tools according to literature procedures.[30] The styrene model was generated by using Dundee Prodrug Server and used as a ligand.[26] The X-ray crystal structure of (*R*)-ibuprofen-bound P450$_{SP\alpha}$ WT was used as a rigid receptor for the docking of styrene. Met-69, Leu-77, Leu-78, and Phe-288, which were located around the hydrophobic and hydrophilic channels, were set as flexible residues for the access of styrene into the active site from the protein surface. For the docking of styrene to the pentanoic-acid-bound-form of P450$_{SP\alpha}$, the model was generated by shortening the alkyl-chain length of palmitic acid in the crystal structure of palmitic-acid-bound WT P450$_{SP\alpha}$. The model was used as a rigid receptor and Met-69, Leu-77, Leu-78, and Phe-288 were set as flexible residues. Docked conformations were ranked automatically by Autodock4 as a free-energy-scoring function. These results were visualized by using PyMOL.[29]

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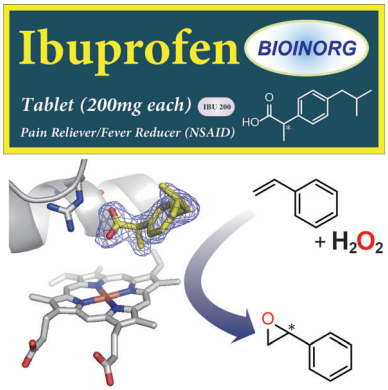
FULL PAPER

The stereoselective epoxidation of styrene was catalyzed by H_2O_2-dependent cytochrome $P450_{SP\alpha}$ in the presence of carboxylic acids as decoy molecules. The stereoselectivity of the reaction could be altered by the nature of the decoy molecules, such as ibuprofen.



Epoxidation

*Takashi Fujishiro, Osami Shoji, Norifumi Kawakami, Takahiro Watanabe, Hiroshi Sugimoto, Yoshitsugu Shiro, Yoshihito Watanabe** ____ ▮▮▮▮–▮▮▮▮

Chiral-Substrate-Assisted Stereoselective Epoxidation Catalyzed by H_2O_2-Dependent Cytochrome $P450_{SP\alpha}$