Crystal Structure of H$_2$O$_2$-dependent Cytochrome P450$_{SP\alpha}$ with Its Bound Fatty Acid Substrate

**INSIGHT INTO THE REGIOSELECTIVE HYDROXYLATION OF FATTY ACIDS AT THE $\alpha$ POSITION**

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Cytochrome P450$_{SP\alpha}$ (CYP152B1) isolated from *Sphingomonas paucimobilis* is the first P450 to be classified as an H$_2$O$_2$-dependent P450. P450$_{SP\alpha}$ hydroxylates fatty acids with high $\alpha$-regioselectivity. Herein we report the crystal structure of P450$_{SP\alpha}$ with palmitic acid as a substrate at a resolution of 1.65 Å. The structure revealed that the C$_{18}$ of the bound palmitic acid in one of the alternative conformation is 4.5 Å from the heme iron. This conformation explains the highly selective $\alpha$-hydroxylation of fatty acid observed in P450$_{SP\alpha}$. Mutations at the active site and the F–G loop of P450$_{SP\alpha}$ did not impair its regioselectivity. The crystal structures of mutants (L78F and F288G) revealed that the location of the bound palmitic acid was essentially the same as that in the WT, although amino acids at the active site were replaced with the corresponding amino acids of cytochrome P450$_{9sB}$ (CYP152A1), which shows $\beta$-regioselectivity. This implies that the high regioselectivity of P450$_{SP\alpha}$ is caused by the orientation of the hydrophobic channel, which is more perpendicular to the heme plane than that of P450$_{9sB}$.

Cytochrome P450s (P450s)$^3$ are ubiquitous heme-containing monoxygenases that play crucial roles in the oxidative metabolism of many exogenous and endogenous compounds (1–4). X-ray crystal structure analysis is one of the most powerful methods for visualizing the structures of P450s and their interactions with substrates in the heme cavity at the atomic level. Since the first crystal structure of P450, P450$_{cam}$ (CYP101A1) was reported by Poulos et al. (5), the crystal structures of P450s from mammals (6–14), archaea (15–17), and bacteria (18–27) have been reported, and interactions between their substrates and amino acid residues at substrate recognition sites have been clarified. Most P450s accomplish monooxygenation by reductive activation of molecular oxygen using NADPH or NADH to produce compound I (oxoferryl porphyrin $\pi$ cation radical). P450s also use H$_2$O$_2$ to generate compound I, but the efficiency of this reaction is poor compared with that of reductive activation of molecular oxygen. In 1994, Matsunaga et al. (28) isolated P450$_{SP\alpha}$ (CYP152B1) from *Sphingomonas paucimobilis* and reported that it exclusively uses H$_2$O$_2$ as the oxidant and catalyzes $\alpha$-selective (100%) hydroxylation of long alkylic chain fatty acids (29). Although P450$_{SP\alpha}$ is the first P450 to be classified as a family of H$_2$O$_2$-dependent P450, its crystal structure has not been determined despite its potential as a biocatalyst. The first crystal structure of H$_2$O$_2$-dependent P450, P450$_{9sB}$ (CYP152A1), which has 44% amino acid identity to P450$_{SP\alpha}$, was reported in 2003 by Lee et al. (30). The crystal structure of a substrate-bound form of P450$_{9sB}$ (Protein Data Bank code 1IZO) revealed that P450$_{9sB}$ lacks general acid-base residues around the distal side of the heme, although this arrangement is highly conserved among peroxidases and peroxygenases (31–35). Instead of the general acid-base residues, the terminal carboxylate group of the bound fatty acid interacts with the guanidinium group of Arg$^{242}$ located near the heme iron. The distance between an oxygen atom of glutamic acid side chain and the heme iron is 5.3 Å, which is similar to that observed in chloroperoxidase (CPO) from *Caldariomyces fumago*; the distance between an oxygen atom of glutamic acid side chain and the heme iron is 5.1 Å (34, 35). The location of the heme is 5.1 Å (34, 35). The location of the heme interacts with the guanidinium group of Arg$^{242}$ located near the heme iron. The distance between an oxygen atom of glutamic acid side chain and the heme iron is 5.3 Å, which is similar to that observed in chloroperoxidase (CPO) from *Caldariomyces fumago*.
of the substrate (Scheme 1). Recently, we have shown that P450<sub>BS</sub>/H9252 is able to catalyze H<sub>2</sub>O<sub>2</sub>-dependent monooxygenation of foreign compounds such as styrene and ethylbenzene in the presence of a carboxylic acid with a short alkyl chain (C<sub>4</sub>–C<sub>10</sub>), a so-called “decoy molecule” (36). The crystal structure of a heptanoic acid (C<sub>7</sub>)-bound form of P450<sub>BS</sub>/H9252 was analyzed, and an interaction between Arg<sup>242</sup> and the carboxylate group of heptanoic acid was detected. We also resolved the crystal structure of the substrate-free form of P450<sub>BS</sub>/H9252 and found that binding of fatty acid or substrate analogues did not induce any notable structural change, whereas the substrate-free form of P450<sub>BS</sub> never reacts with H<sub>2</sub>O<sub>2</sub> (37). These observations further confirm that substrate binding initiates the formation of compound I via a salt bridge between Arg<sup>242</sup> and the carboxylate group at the active site.

P450<sub>BS</sub> oxidizes the α- and β-positions of fatty acids in a 40:60 ratio, whereas P450<sub>SP</sub> exclusively oxidizes the α position. To elucidate the cause of this selectivity, we need to study the crystal structures of both enzymes. Although the crystal structure of the substrate-bound form of P450<sub>BS</sub> is known, that of P450<sub>SP</sub> has not been resolved. Therefore, we crystallized P450<sub>SP</sub> and succeeded in preparing high quality crystals of P450<sub>SP</sub>. Herein we describe the x-ray crystal structure of P450<sub>SP</sub> containing a fatty acid at a resolution of 1.65 Å and examine enzymatic properties of its mutants to study its highly selective α-hydroxylation of fatty acids. We also compare the structure of P450<sub>SP</sub> with that of P450<sub>BS</sub> in the context of similarities and differences among H<sub>2</sub>O<sub>2</sub>-dependent P450s.

**EXPERIMENTAL PROCEDURES**

**Crystallization of P450<sub>SP</sub>**—P450<sub>SP</sub> WT was concentrated to 13.4 mg/ml in 50 mM MES (pH 7.0) containing 20% (v/v) glycerol by centrifugation using Amicon Ultra filter units (Millipore, Co.). A 2-μl aliquot of the concentrated P450<sub>SP</sub> solution was mixed with 2 μl of a reservoir solution composed of 0.1 M HEPES (pH 7.0) and 35% (v/v) MPD. Crystals of P450<sub>SP</sub> were grown by a sitting-drop vapor diffusion method at 20 °C for 6 days. The P450<sub>SP</sub> L78F and F288G mutants were crystallized under the same conditions used for the WT.

**Data Collection, Phasing, and Refinement of P450<sub>SP</sub>**—Crystals were flash-cooled in liquid nitrogen. X-ray diffraction data sets were collected on a beam line BL41XU instrument 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 HKL2000 (38) program was used for integration of diffraction intensities and scaling. Initial phases were calculated and refined using the SHELXE program (39) and the hkl2map graphical interface (40). In the calculated electron density, the main chain was clearly traceable, and the initial polypeptide chain was built using ARP/wARP (41). Model building and refinement were performed using COOT (42), CNS (43), and REFMAC5 (44). TLS refinement (45) was performed in the final stages of the refinement, defining each chain in the asymmetric unit as a separate TLS group. The resulting model had a final R<sub>free</sub> of 15.1% and an R<sub>free</sub> of 17.3% (Table 1). The final model consisted of one polypeptide chain with residues 9–415 of P450<sub>SP</sub>, one heme, one palmitic acid, one MPD, and 371 water molecules. Structure validation was performed using
### Table 1

**Data collection and refinement statistics**

|          | WT       | L78F     | F288G    |
|----------|----------|----------|----------|
| Wavelength (Å) | 1.65     | 1.90     | 1.80     |
| Space group | P3 1.21  | P3 1.21  | P3 1.21  |
| Cell dimensions | a, b, c (Å) | 94.440, 94.440, 113.553 | 94.137, 94.137, 113.402 | 94.58, 94.58, 113.449 |
| Resolution (Å) | 50.00-1.65 | 20.0-1.90 | 20.0-1.80 |
| No. of total observed reflections | 1,516,965 | 495,946 | 582,013 |
| No. of unique reflections | 70,272 | 46,225 | 54,812 |
| Rmerge (%) | 15.1/17.3 | 15.9/18.8 | 16.0/19.0 |
| RMSD bond length (Å) | 0.012 | 0.014 | 0.012 |
| RMSD bond angles (°) | 1.279 | 1.295 | 1.199 |
| No. of atoms | 3749 | 3576 | 3697 |
| Average B-factor (Å²) | 16.8 | 3576 | 3697 |

### Structural Analysis of P450SP

**Hydroxylation of Myristic Acid**

The standard reaction mixture contained 0.1 mM potassium phosphate (pH 7.0), 0–120 mM myristic acid (C14) (0–60 μM for F288G and A172F/F288G), 50 mM P450SP, or P450BS in 200 μM H2O2 in a total volume of 1 ml. The reaction mixture was incubated at 37 °C for 1 min, and then the reaction was quenched by adding 500 μl of dichloromethane followed by vigorous mixing. After the addition of 12-hydroxydocanoic acid as an internal standard, the products were extracted with dichloromethane. For derivatization of the extract, 50 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% (v/v) trimethylchlorosilane (TMCS) was added, and the mixture was incubated in the dark at room temperature for 2 h. The derivatized products were analyzed using a Shimadzu GC-17A (Shimadzu Corp., Kyoto, Japan) equipped with a Shimadzu GC/MS-QP5000 and Rxi™-5ms capillary column (30 m × 0.25 mm; Restek Corp., Bellefonte, PA) to identify the products. The GC/MS analytical conditions were as follows: column temperature, 50 °C (1 min) to 40 °C/min (5 min) to 250 °C (8 min); injection temperature, 250 °C; interface temperature, 280 °C; carrier gas, helium; flow rate, 0.9 ml/min, mode, split mode; and split ratio, 1/50. To quantify the products, derivatization of the extract was performed by adding 9-anthryldiazomethane and incubating the solution in the dark at room temperature for 1 h. For quantification of the products, reverse phase HPLC analysis was performed using an Inertsil® ODS-3 column (4.6 mm × 250 mm; GL Sciences, Inc., Tokyo, Japan) installed on a Shimadzu LC-10AD VP pump systems, a Shimadzu RF-10AXL fluorescence spectrometer, a Shimadzu CTO-10A VP column oven, and a Shimadzu DGU-12A degasser. The HPLC analytical conditions were as follows: flow rate, 1.0 ml/min; acetonitrile/water = 99/1; column temperature, 30 °C; excitation wavelength, 365 nm; emission wavelength, 412 nm; and retention times, 12-hydroxydocanoic acid (6.41 min), β-OH C14 (10.7 min), α-OH C14 (11.9 min), and C14 (21.5 min). Chiral separation of the products was performed on a CHIRALPAK AD-RH column (Daicel Chemical Industries, Ltd., Osaka, Japan) installed on the same reverse phase HPLC system as in the case of the quantification. The absolute configuration was assigned by comparison of the product ratios in the hydroxylation of C14 by P450SP, WT (53) and P450BS, WT (54). The HPLC conditions for the chiral separation were as follows: flow rate, 0.9 ml/min; linear gradient, MeOH/water = 85/15 (0–10 min) to 100/0 (100–120 min); column temperature, 40 °C; excitation, 365 nm; emission, 412 nm; retention times, (R)-α-OH C14 (34.2 min), (S)-α-OH C14 (37.6 min), (S)-β-OH C14 (49.8 min), and (R)-β-OH C14 (53.2 min).

**UV-visible and EPR Measurements**—UV-visible spectra were recorded using a Shimadzu UV-2400 PC spectrophotometer at room temperature. X-band EPR spectra were recorded using an E500 X-band CW-EPR instrument (Bruker, Ettlingen, Germany) at 10 K. A cryostat (ITC503; Oxford Instruments Co., Abingdon, UK) was used for measurements at low temperatures.
RESULTS AND DISCUSSION

Overall Structure and Substrate Binding—The structure of P450SP/H9251 was resolved at a resolution of 1.65 Å (Fig. 1). One molecule was observed in the asymmetric unit. The overall structure exhibited typical P450 folding with 17 α helices and three β sheets. It has a trigonal prism-shaped structure with the heme buried deep inside the protein. The I helix lays across the interior of the P450 molecule on the distal side of the heme group. Two channels connecting the active site cavity with the protein surface were identified (Fig. 1). Channel I is composed of hydrophobic residues (Ile73, Leu77, Leu78, Phe169, Ala172, Ala245, Phe287, Phe288, Pro289, Leu398, and Pro399) (Fig. 2A). Channel II includes hydrophilic residues (Gln84 and Asn238) as its constituent residues. A cluster of water molecules with a hydrogen-bonding network was observed in Channel II (Fig. 2D). We expect that Channel II would be used for the ingress of H₂O₂ and the egress of water during the reaction. Phe288 is located at the border of the two channels, but the two channels are not clearly separated because the entrances of the channels are wide (Figs. 1, B and C, and 3A).

Although no substrates were added to the purified P450SP, the initial 2Fo – Fc electron density map showed a long continuous electron density in Channel I (Fig. 2A). One of the ends of this electron density, located near Arg241 in the active site, has a Y-shape. Because the shape of this electron density is very similar to that of a long alkyl chain fatty acid observed in P450BM3 (21) and P450Hsp (30), we assumed that this electron density corresponds to a long alkyl chain fatty acid originating from Escherichia coli cells (55). Indeed, GC/MS analysis of the extract of the purified P450SP with dichloromethane showed that palmitic acid and stearic acid were coexistent, even after purification (supplemental Fig. S1). It was difficult to deduce the length of the alkyl chain of the fatty acid based on the electron density of the substrate(s) because the electron density of the substrate was shorter than the alkyl chain of palmitic acid, possibly because of disordering. Therefore, we tentatively assigned this electron density to palmitic acid. In addition, because the Y-shaped electron density adjacent to the Arg241 was accompanied by an additional electron density, two alternative conformations with occupancies of 0.7 (Conformation A) and 0.3 (Conformation B) were placed and refined (Fig. 2, B and C). The terminal alkyl chain of palmitic acid in the final structure is highly disordered, indicating that the terminal alkyl chain is loosely fixed. It is noteworthy that the A’-helix, B’-he-
lix, and F–G loop have relatively high B-factors (Fig. 3B) and that the entrance of Channel I is open wide (Fig. 3A), suggesting that this region is flexible even though the substrate was accommodated. The substrate access channel and B-factor of P450BSI/H9252 are shown in supplemental Fig. S2 for comparison.

The charge distribution on the surfaces of the proximal and distal sides of P450SPα is shown in Fig. 4. In contrast to the charge distribution typical of P450s such as P450BM3 (56, 57), negative surface charges were observed on the proximal side of P450SPα. A positive surface potential on the proximal side of regular P450 is important for the recognition of reductases in the electron transfer step of oxygen activation in the P450 catalytic cycle (58). The negative surface potential on the proximal side of P450SPα may preclude binding of a reductase. This unique charge potential distribution of P450SPα is an indication that P450SPα does not need binding of a reductase and prefers...
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Active Site Structure—At the active site, Arg$^{241}$ is located above the heme, and its guanidinium group interacts with the carboxylate group of palmitic acid (Fig. 2A); the distances between two oxygen atoms of the carboxylate group and the guanidinium group of Arg$^{241}$ (N$_{y2}$ and N$_{y}$) are 2.9 and 3.0 Å in Conformation A (Fig. 2B) and 3.3 and 3.2 Å in Conformation B (Fig. 2C), respectively. In contrast to other heme enzymes that utilize $\text{H}_2\text{O}_2$, P450$_{\text{SP}}$ lacks general acid-base residues around the distal side of the heme. As an alternative, the terminal carboxylate group of palmitic acid is located above the heme. The distance between the oxygen atoms close to the heme iron is 5.2 Å for Conformation A and 5.5 Å for Conformation B, indicating that the location of the oxygen atom is similar to that of the glutamic acid moiety of CPO (34, 35) and that of the terminal carboxylate group of palmitic acid observed in P450$_{\text{BS}^\beta}$. These observations indicate that participation of the terminal carboxylate group of the fatty acid in the generation of active species using $\text{H}_2\text{O}_2$ (Scheme 1) is common among $\text{H}_2\text{O}_2$-dependent P450s. The distal side of the heme is hydrophilic because of Gln$^{84}$, Asp$^{238}$, Arg$^{241}$, and the carboxylate group of palmitic acid (Fig. 2D). The polar environment is expected to facilitate the heterolytic cleavage of the O–O bond of $\text{H}_2\text{O}_2$ to generate compound I, as is proposed for heme peroxidases such as cytochrome c peroxidase (31, 32), HRP (33), CPO (34, 35), and myoglobin mutants (60, 61). A water molecule is located 2.1 Å away from the heme iron and could function as a sixth ligand on the heme even though the palmitic acid occupies the distal side of the heme. As an alternative, the terminal carboxylate group of palmitic acid is located above the heme. The distance between the oxygen atoms close to the heme iron is 5.2 Å for Conformation A and 5.5 Å for Conformation B, indicating that the location of the oxygen atom is similar to that of the glutamic acid moiety of CPO (34, 35) and that of the terminal carboxylate group of palmitic acid observed in P450$_{\text{BS}^\beta}$. These observations indicate that participation of the terminal carboxylate group of the fatty acid in the generation of active species using $\text{H}_2\text{O}_2$ (Scheme 1) is common among $\text{H}_2\text{O}_2$-dependent P450s. The distal side of the heme is hydrophilic because of Gln$^{84}$, Asp$^{238}$, Arg$^{241}$, and the carboxylate group of palmitic acid (Fig. 2D). The polar environment is expected to facilitate the heterolytic cleavage of the O–O bond of $\text{H}_2\text{O}_2$ to generate compound I, as is proposed for heme peroxidases such as cytochrome c peroxidase (31, 32), HRP (33), CPO (34, 35), and myoglobin mutants (60, 61). A water molecule is located 2.1 Å away from the heme iron and could function as a sixth ligand on the heme even though the palmitic acid occupies the distal side of the heme cavity (Fig. 2A). The UV-visible spectra of P450$_{\text{SPa}}$ in the absence and presence of 120 μM of myristic acid showed a Soret absorption peak at 417 nm, which is consistent with a typical six-coordinate low-spin ferric heme (Fig. 6). The EPR spectrum of the substrate-free form of P450$_{\text{SPa}}$ showed a ferric low spin state having g value 2.59 ($g_x$), 2.25 ($g_y$), and 1.85 ($g_z$) (Fig. 7), suggesting that the electronic environment of the heme iron of P450$_{\text{SPa}}$ resembles that of CPO (2.61 ($g_x$), 2.26 ($g_y$), 1.83 ($g_z$))
(g), pH 5.2) (62) rather than those of bacterial P450s such as P450cam (2.45 (g), 2.26 (g), 1.91 (g)) (63) and P450BM3 (2.42 (g), 2.26 (g), 1.92 (g)) (64). The EPR spectral change of P450sp\textsubscript{a} upon addition of myristic acid (120 \mu M) was very small, indicating that the low spin state is essentially retained irrespective of substrate binding. Minor signals at g = 2.67 in the substrate-free form and at g = 2.53 in the myristic acid-bound form might reflect P420 species of P450sp\textsubscript{a} (supplemental Fig. S5), whereas signals are different from that of P420 species of P450cam (2.46 (g)) (64).

Regioselectivity for Hydroxylation of Fatty Acid—P450sp\textsubscript{a} exclusively catalyzes the hydroxylation of fatty acid at the C\textsubscript{\alpha} position and produces the corresponding \alpha-hydroxy fatty acid, whereas P450bsb produces \alpha and \beta hydroxy products in the ratio of 43:57. In the crystal structure of P450sp\textsubscript{a}, the distances of the C\textsubscript{\alpha} carbon and the C\textsubscript{\beta} carbon in Conformation B from the heme iron are 4.5 and 5.5 Å, respectively. Because the C\textsubscript{\alpha} carbon in Conformation B is clearly close to the iron atom, and the distance of 4.5 Å agrees well with the distance between the C5 position of \alpha-d-camphor and the heme iron in P450cam (65), Conformation B is expected to produce the \alpha-hydroxy fatty acid selectively. Because the C\textsubscript{\alpha} and C\textsubscript{\beta} carbons in Conformation A are both far away from the heme iron in respect of the hydroxylation reaction, we assume that Conformation A is a nonproductive conformation. In the crystal structure of P450bsb, the C\textsubscript{\alpha} and C\textsubscript{\beta} carbons of palmatic acid are located at distances of 5.0 and 6.2 Å from the heme iron, respectively. The substrate observed in P450bsb needs to be closer to the heme iron to be hydroxylated, as was observed for P450BM3 (66). The C\textsubscript{\alpha} and C\textsubscript{\beta} carbons of the possible productive conformation of palmatic acid in P450bsb may be equally close to the heme iron.

Structural Comparison of P450sp\textsubscript{a} with P450bsb—To gain further insight into \textsubscript{H2}O\textsubscript{2}-dependent P450s, the structure of P450sp\textsubscript{a} was compared with that of P450bsb. The structure of P450sp\textsubscript{a} is superimposed on that of P450bsb in Fig. 8. Except for the B helix and the F–G loop, the overall structures are well superposed with a root mean square deviation value of 1.4 Å. The locations of Channel I are notably different, resulting in different locations for the bound palmitic acid. The palmatic acid is more perpendicular to the heme plane in P450sp\textsubscript{a} than in P450bsb. The active site cavity of P450sp\textsubscript{a} is smaller than that of P450bsb (supplemental Fig. S3). The smaller active site cavity is mainly due to the presence of Phe\textsuperscript{288} in the heme cavity of P450sp\textsubscript{a}. The corresponding amino acid residue in P450bsb is Gly\textsuperscript{290}. The effect of Phe\textsuperscript{288} and the location of Channel I are discussed in the next section.

Hydroxylation of Myristic Acid by Mutants—We carried out mutagenesis study to elucidate the structural requirement for the \alpha-selective reaction of P450sp\textsubscript{a}. Based on the comparison between amino acid residues in the active sites of P450sp\textsubscript{a} and P450bsb, two amino acid residues in the active site of P450sp\textsubscript{a}, Phe\textsuperscript{288} and Leu\textsuperscript{78}, were mutated to the corresponding P450bsb residues and vice versa. The active site cavity of P450sp\textsubscript{a} is smaller than that of P450bsb mainly because of the side chain of Phe\textsuperscript{288} (supplemental Fig. S3), which interacts directly with the fatty acid. Although Leu\textsuperscript{78} does not interact with the fatty acid, it is located at the border of the channels and may affect the catalytic reaction. Three mutants of P450sp\textsubscript{a}, L78F, F288G, and L78F/F288G, and three P450bsb mutants, F79L, G290F, and F79L/G290F, were prepared and their catalytic activities, regioselectivities, and stereoselectivities were examined (Table 2). P450bsb mutants F79L and G290F had 75 and 95% \alpha selectivity,
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### TABLE 2

| Enzyme          | \( k_{\text{cat}} \) | \( K_m \) | Regioselectivity | Stereoselectivity |
|-----------------|---------------------|----------|-----------------|------------------|
|                 | nmol/min/nmol P450 | \( \mu \text{M} \) | \( \alpha-\text{OH} \% \) | At \( \alpha \)-position (R):(S) | At \( \beta \)-position (R):(S) |
| P450<sub>Sp</sub> WT | 1300 ± 20         | 43 ± 2   | >99             | 3:97             | 4:96             |
| P450<sub>Sp</sub>L78F | 5500 ± 930       | 300 ± 70 | >99             | 19:81            | 3:97             |
| P450<sub>Sp</sub>F288G | 770 ± 70        | 100 ± 20 | >99             | 19:81            | 3:97             |
| P450<sub>Sp</sub>L78F/F288G | 1300 ± 40    | 85 ± 16  | >99             | 21:79            | 4:96             |
| P450<sub>Sp</sub>A172F | 1200 ± 20      | 68 ± 3   | >99             | 17:83            | 98:2             |
| P450<sub>Sp</sub>A172F/F288G | 100 ± 2         | 23 ± 2   | >99             | 76:24            | 99:1             |
| P450<sub>Sp</sub>WT | 1400 ± 180       | 66 ± 20  | 43              | 62:38            | 79:1             |
| P450<sub>Sp</sub>F79L | 1400 ± 30        | 54 ± 3   | 75              | 29:71            | 85:15            |
| P450<sub>Sp</sub>G290F | 1900 ± 210      | 190 ± 30 | 95              | 28:72            | 86:14            |
| P450<sub>Sp</sub>F79L/G290F | 890 ± 20       | 95 ± 3   | 77              | 99:4:96          | 99:4:96          |

respectively, indicating that the regioselectivity of P450<sub>Spβ</sub> was greatly altered. The substitution of Gly<sup>290</sup> with Phe must induce a conformational change in myristic acid, and the C<sub>α</sub> carbon is expected to move closer than the C<sub>β</sub> carbon to the heme iron. In sharp contrast with the P450<sub>Spα</sub> mutants, no clear differences in regioselectivity were observed for P450<sub>Spα</sub> mutants, whereas the amino acids at the active site were replaced with the corresponding amino acids of P450<sub>Spβ</sub> which has β-regioselectivity. Three mutants of P450<sub>Spα</sub> showed >99% α selectivity. To further investigate the regioselectivity of P450<sub>Spα</sub>, two mutants of P450<sub>Spα</sub>, A172F and A172F/F288G, were prepared. Ala<sup>172</sup> located in the F–G loop seems to be important for controlling fatty acid conformation. However, the regioselectivity was not affected by these mutagenesis experiments, indicating that the mutagenesis at the active site and at the F–G loop does not alter the position of bound palmitic acid. The x-ray crystal structures of the L78F and F288G mutants revealed that there is little difference in the location of the bound palmitic acid in Conformation B (the productive conformation) and the location of the hydrophilic channel (Fig. 9), whereas the position of the amino acids located near the active site, Met<sup>69</sup>, Pro<sup>289</sup>, and Pro<sup>389</sup>, are shifted (Fig. 10 and supplemental Fig. S4). The C<sub>α</sub> positions of palmitic acid in both mutants are essentially the same as in the WT. Whereas the stereoselectivity of α hydroxylation of myristic acid was slightly reduced by mutagenesis (Table 2), the regioselectivity of P450<sub>Spα</sub> was not affected, suggesting that the small perturbation induced by the mutagenesis is insufficient to alter the high regioselectivity of P450<sub>Spα</sub>. We presume that the regioselectivity of P450<sub>Spα</sub> is highly controlled by its hydrophobic channel. The orientation of the channel (almost perpendicular to the heme plane) and the wide open entrance appear to be crucial for the high α-selectivity of hydroxylation. The hydrophobic channel may control the direction of the fatty acid access. Further mutagenesis studies, especially at the F and G helices and at the F–G loop region, are necessary for elucidating the mechanistic details of the highly selective α-hydroxylation of fatty acid catalyzed by P450<sub>Spα</sub>.

**Conclusion**—We have determined the x-ray crystal structure of \( \text{H}_2\text{O}_2 \)-dependent P450<sub>Spα</sub> as a palmitic acid-bound form at a resolution of 1.65 Å. The crystal structure revealed that the carboxylate group of the fatty acid interacts with Arg<sup>281</sup>, which is located above the heme. Previous studies on the reaction mechanism of P450<sub>Spβ</sub> suggested that the carboxylate group of the fatty acid serves as a general acid-base catalyst for the generation of compound I using \( \text{H}_2\text{O}_2 \). Our crystal structure study confirms that this substrate-assisted activation mechanism is also conserved in P450<sub>Spα</sub>, indicating that the substrate-assisted activation mechanism is common in the \( \text{H}_2\text{O}_2 \)-dependent P450-catalyzed hydroxylation reaction with fatty acids. Notably, a water molecule was observed as the sixth ligand of the heme iron, even in the presence of palmitic acid. Consistent
with the crystal structure, the ferric low spin state of P450_{Spd} was retained irrespective of the substrate binding. These results also indicate that the shift in redox potential of the heme that is induced by substrate binding, which is generally indispensable for the reductive activation of molecular oxygen, is not essential for the H$_2$O$_2$-dependent P450s. Crystallographic studies on substrate binding revealed that the C$_9$ carbon of the bound palmitic acid in Conformation B is situated close to the heme iron (4.5 Å). This conformation explains the highly selective α-hydroxylation of fatty acid. Surprisingly, mutations at the active site and at the F–G loop of P450_{Spd} did not impair the high regioselectivity. The crystal structures of the L78F and F288G mutants revealed that the location of the bound palmitic acid was not affected by these mutations. These results imply that the orientation of the hydrophobic channel of P450_{Spd}, which is more perpendicular to the heme plane than that of P450_{Smp}, is crucial for the highly selective α-hydroxylation. Although further mutagenesis studies are required to fully understand the high regioselectivity of P450_{Spd}, the structural studies reported here contribute to a better understanding of the relationship between the structure and function of H$_2$O$_2$-dependent P450s at the atomic level.

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