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Unique Heme Environment at the Putative Distal Region of Hydrogen Peroxide–Dependent Fatty Acid α-Hydroxylase from *Sphingomonas paucimobilis* (Peroxygenase $P450_{SP\alpha}$)

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Fatty acid α-hydroxylase from *Sphingomonas paucimobilis* is a hydrogen peroxide-dependent cytochrome P450 (P450) enzyme ($P450_{SP\alpha}$). In this study, heme-ligand exchange reactions of $P450_{SP\alpha}$ were investigated using the optical spectroscopic method and compared with those of various P450s. Alkylamines ($C \geqq 5$) induced changes in the spectrum of ferric $P450_{SP\alpha}$ to one typical of a nitrogenous ligand-bound low-spin form of ferric P450, although their affinities were lower than those for other P450s, and a substrate, laurate, did not interfere with the binding in contrast with in the cases of other P450s. Other compounds having a nitrogen donor atom to the heme iron of P450, including pyridine or 1-methylimidazole, induced no change in the spectrum of $P450_{SP\alpha}$ in either the ferric or ferrous state. Practically no spectral change was observed on the addition of alkyl isocyanides to ferric P450s. On the other hand, cyanide induced a change in the spectrum of ferric $P450_{SP\alpha}$ to one characteristic of cyanide-bound form of ferric P450. The affinity of cyanide increased when the substrate was added, in contrast with in the cases of other P450s. Ferrous $P450_{SP\alpha}$ combined with CO and alkyl isocyanides, and the affinity for CO was of the same order of magnitude as in the cases of other P450s. These findings suggest a unique heme environment of $P450_{SP\alpha}$, in which most compounds usually acting as external ligands of ferric P450s are prevented from gaining access to the heme iron of $P450_{SP\alpha}$. The unique properties of the hydroxylase reaction catalyzed by $P450_{SP\alpha}$, where an oxygen atom of hydrogen peroxide but not of molecular oxygen is utilized and incorporated into a fatty acid at its α position, is possibly related with such a specific heme environment of this P450. A possible mechanism for the peroxygenase reaction of $P450_{SP\alpha}$ is proposed.

Key words: cytochrome P450, fatty acid α-hydroxylase, heme-ligand interaction, peroxygenase, *Sphingomonas paucimobilis*.

Fatty acid α-hydroxylase purified from *Sphingomonas paucimobilis*, a 2-hydroxymyristic acid–containing sphingolipid-rich bacterium, is a unique enzyme, peroxygenase, which introduces an oxygen atom from hydrogen peroxide into myristic acid and other fatty acids to produce the corresponding 2-hydroxy fatty acids (*6*, *7*). The gene of the α-hydroxylase was recently cloned and expressed, and the recombinant enzyme was purified so as to be characterized (*2*). Sequence analysis of the gene revealed that the α-hydroxylase was a novel member of the cytochrome P450 (P450) superfamily and thus the α-hydroxylase was given $P450_{SP\alpha}$ as a trivial name (*3*). The recombinant enzyme was confirmed to show a CO difference spectrum characteristic of P450s, although the α-hydroxylase activity was insensitive to carbon monoxide (*7*).

Although $P450_{SP\alpha}$ has amino acid sequences highly conserved in the members of the P450 superfamily, which are associated with their secondary structures (*8*), the sequence of the putative distal helix of $P450_{SP\alpha}$ shows no significant homology to those of monooxygenase P450s, and $P450_{SP\alpha}$ lacks the conserved threonine in this helix (*2*). The groove formed in this helix at the conserved threonine provides a pocket for molecular oxygen (*9–12*). In addition, the consensus motif of the heme-binding region is modified, where the conserved phenylalanine is substituted with proline, and a seven amino acid insertion is found between conserved glycine and cysteine residues (*2*). Similar modifications are found in the sequences of P450s (*3*, *13–16*), which do not use molecular oxygen for their enzyme activities but catalyze peroxide-related reactions. $P450_{BS\beta}$ [the *ybdT* gene product of *Bacillus subtilis* (*17*)], which exhibits 44% identity in the deduced amino acid sequences to $P450_{SP\alpha}$, has the hydrogen peroxide-dependent α- and β-hydroxylase activity of myristic acid (*3*). Allene oxide synthase (CYP74A) (*13*) and fatty acid hydroperoxide lyase (CYP74B) (*14*) utilize peroxy substrates, while thromboxane synthase

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The nomenclature for cytochrome P450 is adopted from Nelson *et al.* (*1*). Abbreviations: P450, cytochrome P450; $P450_{SP\alpha}$, product of the *CYP152B1* gene (*2*, *3*); $P450_{BS\beta}$, product of the *CYP152A1* gene (*3*); P450cam, product of the *CYP101* gene (*4*); P450nor, product of the *CYP55* gene (*5*); MC, 3-methylcholanthrene.

(CYP5A1) (*15*) and prostacyclin synthase (CYP8A1) (*16*) metabolize an endoperoxide. Thus, we assume that P450s catalyzing peroxide-related reactions have unique heme environments compared with usual monooxygenase P450s.

It has been proposed, based on a comparison of the crystal structures and sequence alignments of various P450s, that the ternary structure of the heme pocket may be generally conserved in all P450s but will differ in many details among the various P450s (*8–12*, *18*, *19*). Despite the good conservation of sequences in the proximal and distal heme regions, clear divergence among the distal helices has actually been shown in the P450 crystal structures known (*11*, *12*). The crystal structures have been determined for six P450s (*9*, *10–12*, *18*, *19*), but those of P450s catalyzing peroxide-related reactions have not yet been solved.

Heme-ligand exchange reactions have been utilized to inspect the heme environments of hemoproteins and individual differences in the exchange reactions have been reported among P450 species (*20–25*). In this study, we performed spectral analyses to examine the interactions of various external ligands with $P450_{SP\alpha}$ and compared them with those with other P450s (P450cam, P450nor, P4502C2, P4502E1, P4501A2, and P4502B4). P450cam is the most extensively studied P450 species. P450nor is not a monooxygenase but reduces nitric oxide directly with NADH, and its crystal structure has been determined. P4502C2 and P4502E1 were adopted because both P450s can hydroxylate fatty acids at the ω-1 position. P4501A2 and P4502B4 are xenobiotic-metabolizing P450s, the heme-ligand interactions of which have been extensively studied. The interactions of various ligands with $P450_{SP\alpha}$ were anomalous in the ferric state but rather normal in the ferrous state, suggesting a unique heme environment of $P450_{SP\alpha}$ where most possible nitrogenous ligands for P450 can not gain access to the ferric heme of $P450_{SP\alpha}$. The ferrous state is unlikely to be involved in the mechanism of the peroxygenase reaction of $P450_{SP\alpha}$.

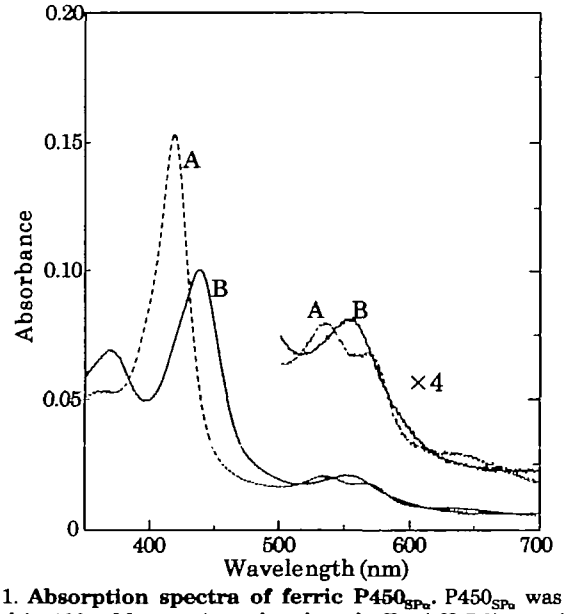

Fig. 1. **Absorption spectra of ferric $P450_{SP\alpha}$.** $P450_{SP\alpha}$ was dissolved in 100 mM potassium phosphate buffer (pH 7.0) containing 20% glycerol. Spectra were measured in the absence (A) and presence of 10 mM potassium cyanide (B).

MATERIALS AND METHODS

$P450_{SP\alpha}$ was expressed in transformed *Escherichia coli* cells and the recombinant $P450_{SP\alpha}$ was purified, as previously described (*2*, *26*). P450cam and P450nor were prepared from transformed *E. coli* cells according to the published procedures (*27*, *28*). P4502C2 and P4502E1 were prepared from transformed yeast cells, as previously described (*29*, *30*). P4501A2 and P4502B4 were purified from rabbit liver microsomes, as previously described (*31*). *t*- and *n*-Butyl isocyanides were purchased from Aldrich Chemical (Milwaukee, WI, USA). Ethyl isocyanide was synthesized by the method of Jackson and McKusick (*32*). Other chemicals used were from the same sources as previously described (*24*) or of the highest quality commercially available.

Spectrophotometric measurements were carried out with a Jasco Ubest 50 spectrophotometer at room temperature.

RESULTS

Interaction of Cyanide with $P450_{SP\alpha}$—The absorption spectrum of ferric P450 characterizes the bonding atom of the sixth ligand to its heme iron (*20–22*). Ferric $P450_{SP\alpha}$ gave a low-spin type spectrum of a P450 exhibiting absorption maxima at 568, 536, 418, and 363 nm (Fig. 1A), but the spectrum was a little deviated from a typical one in the visible region (the β band was more intense than the α band). When cyanide, which inhibited the activity of $P450_{SP\alpha}$ (*7*), was added to ferric $P450_{SP\alpha}$, the spectrum changed to that of a typical cyanide complex of ferric P450 (Fig. 1B): the Soret band was seen at 439 nm together with a distinct δ band (λ_{max}, 366 nm), and the α band was observed as a shoulder on the β band (λ_{max}, 552 nm). The dissociation constant (*K*s) of cyanide was determined from the spectral titration of $P450_{SP\alpha}$ with potassium cyanide. The affinity depended on the buffer used, but the *K*s value was in the range of 0.9 mM (potassium phosphate, pH 7.0) to 0.3 mM (Tris-HCl, pH 8.0). These values were lower than those estimated for microsomal P450s which metabolize fatty acids or hydrocarbons (Table I).

Interaction of Nitrogenous and Oxygenous Ligands with $P450_{SP\alpha}$—Pyridine and imidazole derivatives are usually used as standard external ligands of P450 in ligand replacement reactions (*20–24*). However, when pyridine (124 mM), 1-methylimidazole (42 mM), or metyrapone (4 mM) was added to $P450_{SP\alpha}$, the absorption spectrum did not change in either the ferric or ferrous state. No spectral change was observed on the addition of 2-, 3-, or 4-methylpyridine, or 2- or 4-methylimidazole.

TABLE I. **Affinity of KCN for various P450s.**

Species	*K*s (mM)		
	Substrate		
	−	+	Substrate, concentration
$P450_{SP\alpha}$	0.9	0.3	Sodium laurate, 0.1 mM
P450cam	0.6	6	Camphor, 0.1 mM
P450nor	0.3	n.e.[a]	
P4502C2	45	70	Sodium laurate, 0.2 mM
P4502E1	15	30	Sodium laurate, 0.2 mM
P4501A2	20	∞[b]	MC-bound form
P4502B4	7[c]	n.e.	

[a]Not examined. [b]Not bound. [c]Ref. *22*.

When 1-octylamine was added to ferric $P450_{SPα}$, an absorption spectrum characteristic of the nitrogenous ligand-bound low-spin form of ferric P450 was observed: a Soret peak was red-shifted, as compared with for the native form of $P450_{SPα}$, to 424 nm (Fig. 2A), and the β band ($λ_{max}$, 538 nm) became clearly more prominent than in the case of the native form. The *Ks* value of 1-octylamine was determined from the spectral titration of $P450_{SPα}$ (Table II), and was considerably lower than those reported for other P450s (*21*, *22*). Similar spectral changes were observed on the binding of 1-heptylamine, 1-hexylamine, and 1-pentylamine to ferric $P450_{SPα}$, but the *Ks* value decreased as the length of the alkyl chain decreased (Table III). No spectral change was observed on the addition of 1-butylamine (400 mM).

When the 1-pentylamine or 1-hexylamine complex of ferric $P450_{SPα}$ was reduced with dithionite, the spectrum changed to that of the external ligand-free form of ferrous $P450_{SPα}$ (typical pentacoordination form of ferrous P450), indicating that neither alkylamine bound ferrous $P450_{SPα}$ at the concentration of 100 mM. When the 1-octylamine complex was examined, a small but clear band was observed at around 450 nm, together with the band due to the pentacoordination state (Fig. 2B). A faint shoulder was seen at around 450 nm in the case of the 1-heptylamine complex.

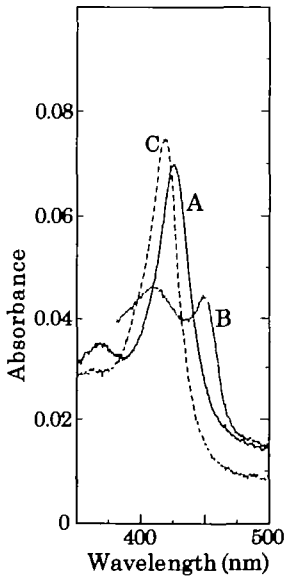

Fig. 2. **Absorption spectra of the 1-octylamine complex of $P450_{SPα}$.** $P450_{SPα}$ was dissolved in 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol and 0.4% sodium cholate. Spectra were measured in the presence of 45 mM 1-octylamine. A, oxidized form; B, dithionite-reduced form; C, oxidized form in the absence of 1-octylamine.

TABLE II. **Affinities of 1-hexylamine and 1-octylamine for various P450s.**

Species	*Ks* (mM)				
	1-Octylamine		1-Hexylamine		
	Substrate				
	−	+	−	+	Substrate, concentration
$P450_{SPα}$	23	12	120	130	Sodium laurate, 0.1 mM
P450cam	0.04	1.5	0.2	16	Camphor, 0.1 mM
P450nor	15	n.e.[a]	100	n.e.[a]	
P4502C2	0.03	0.4	0.9	7	Sodium laurate, 0.2 mM
P4502E1	0.02	0.01	0.07	0.07	Sodium laurate, 0.2 mM
P4501A2	2	∞[b]	12	∞[b]	MC-bound form
P4502B4	0.02[c]	n.e.	0.7	1	Benzphetamine, 1 mM

[a]Not examined. [b]Not bound. [c]Ref. *22*.

The addition of alcohols, such as 1-propanol and 1-butanol, to ferric P450 often induces changes in its spectrum (*20–24*). However, these alcohols (100 mM) did not affect the spectrum of ferric $P450_{SPα}$. When hydrogen peroxide (150 μM) was added to ferric $P450_{SPα}$, no spectral change was detected.

Interaction of CO with Ferrous $P450_{SPα}$—The fatty acid α-hydroxylase activity of $P450_{SPα}$ is not affected when 80% of CO (by volume) is used as the gas phase in the reaction (*7*). However, when CO is thoroughly bubbled through to ferrous $P450_{SPα}$, the spectrum of the typical CO complex of P450 is observed, indicating that CO can bind to $P450_{SPα}$ (*2*). Thus, the affinity of CO for ferrous $P450_{SPα}$ was examined by means of spectral titration of $P450_{SPα}$ with CO. The *Ks* value estimated (Table IV) was comparable to those reported for other P450s (*25*, *33*, *34*). These findings suggest that the ferrous form of $P450_{SPα}$ is not involved in the mechanism of the α-hydroxylation reaction catalyzed by this P450.

Interaction of Alkyl Isocyanides with $P450_{SPα}$—The alkyl isocyanide complexes of most P450s, in the ferrous state, exist in two interconvertible states (the 455 and 430 nm states), which are in pH-dependent equilibrium, and the relative amounts of the two states under a specific condition depend on the P450 species, probably reflecting the differences in the heme environment of each P450 (*24*). The *n*-butyl isocyanide complex of ferrous $P450_{SPα}$ gave a single peak at 452 nm with no detectable band at around 430 nm in the Soret region and an intense β band at 545 nm with a shoulder (α band) at 570–575 nm in the visible region. The spectrum did not change over the pH range of 6.0 to 8.0. The *t*-butyl and ethyl isocyanide complexes of ferrous $P450_{SPα}$ showed quite similar spectra to the *n*-butyl isocya-

TABLE III. **Affinities of various alkylamines for $P450_{SPα}$.**

Alkylamine	*Ks* (mM) Substrate (sodium laurate, 1 mM)	
	−	+
1-Octylamine	23	12
1-Heptylamine	90	60
1-Hexylamine	120	130
1-Pentylamine	>400	n.e.[a]
1-Butylamine	∞[b]	n.e.

[a]Not examined. [b]Not bound at 400 mM.

TABLE IV. **Affinity of CO for various P450s.**

Species	*Ks* (mM) Substrate			Reference
	−	+	Substrate, concentration	
$P450_{SPα}$	0.5	0.4	Sodium laurate, 0.1 mM	
P450cam	0.5	4		*33*
P450nor	10	n.e.[a]		*34*
P4502C2	0.4	1	Sodium laurate, 0.2 mM	
P4501A2	1	10		*25*
P4502B4	0.3–0.5	0.3–0.5		*25*

[a]Not examined.

TABLE V. **Affinities of alkyl isocyanides for ferrous P450s.**

Species	*Ks* (mM)		
	Ethyl isocyanide	*t*-Butyl isocyanide	*n*-Butyl isocyanide
$P450_{SPα}$	0.7	0.5	0.04
P450cam	0.009	0.06	0.01
P450nor	0.3	0.4	0.03
P4502C2	0.002	0.02	0.008
P4502E1	0.003	0.002	0.002
P4501A2	0.02	0.02	0.005
P4502B4	0.004	0.003	0.003

Ks values for P450s other than $P450_{SPα}$ are cited from Ref. *24*.

nide complex and the spectral features resembled those of the alkyl isocyanide complexes of P450cam (*24*, *35*). The *Ks* values of alkyl isocyanides for ferrous $P450_{SPα}$ were estimated from the spectral titration of ferrous $P450_{SPα}$ with alkyl isocyanides. $P450_{SPα}$ always showed lower affinities for alkyl isocyanides than other P450s, when compared for the same alkyl isocyanide (Table V). The *Ks* value of *n*-butyl isocyanide for $P450_{SPα}$ was smaller by one order of magnitude than those of *t*-butyl and ethyl isocyanides.

When alkyl isocyanides are bound to ferric P450, absorption spectra with a Soret peak at around 430 nm are observed, although the affinities of alkyl isocyanides for ferric P450 are lower than those for ferrous P450 (*24*). However, only a slight change in the spectrum was induced when 4 mM *n*-butyl isocyanide was added to ferric $P450_{SPα}$, and no spectral change was observed in the case of *t*-butyl isocyanide.

Effects of Substrates on Ligand Interactions with $P450_{SPα}$—The binding of external ligands to P450 heme is often interfered with by the substrate of P450 monooxygenase and its analogues (*25*, *33*, *35*, *36*). Thus, the effects of fatty acids on the ligand interaction with $P450_{SPα}$ were examined and compared with those in the cases of other P450s. Although the *Ks* value of cyanide for $P450_{SPα}$ changed depending on the buffer, as mentioned above, it always decreased on the addition of laurate, a substrate of fatty-acid α-hydroxylase $P450_{SPα}$ (*7*). As $P450_{SPα}$ can hydroxylate C_{11}–C_{18} fatty acids (*7*), the *Ks* of cyanide was determined in the presence of various fatty acids to examine the effect of the carbon chain length of the fatty acid on the cyanide binding. The *Ks* value changed as a function of the carbon chain length and laurate was found to be the most effective (Fig. 3). It seems that fatty acids with an optimal carbon chain length facilitated the binding of cyanide to $P450_{SPα}$ heme. Myristate was not sufficiently soluble even at 50 μM (half the concentration examined for C_8–C_{13} fatty acids) under the titration conditions, and therefore fatty acids with an alkyl chain larger than that of myristate were not examined. It was confirmed that, in contrast with $P450_{SPα}$, the substrates interfered with the binding of cyanide in the cases of other P450s (Table I). Hydrogen peroxide (150 μM) did not affect the affinity of cyanide for ferric $P450_{SPα}$.

The apparent affinity of octylamine to $P450_{SPα}$ also increased with the addition of the substrate, laurate, in contrast with other P450s (Table II). Similarly, the substrate binding interferes with the CO binding to P450 heme in most cases (*25*, *33*), but not in the case of $P450_{SPα}$ (Table IV). These findings suggest that the binding of fatty acid substrates with an optimal carbon chain length does not interfere with the access of the external ligand molecules to the heme iron of $P450_{SPα}$ but rather assists it.

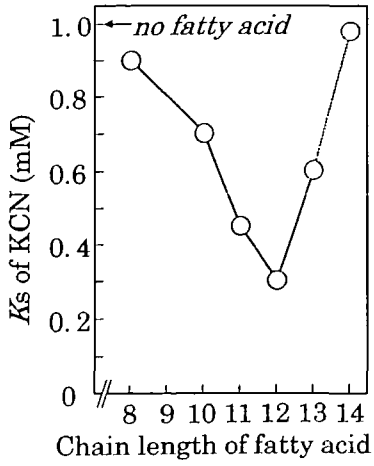

Fig. 3. **Effects of fatty acids on the affinity of cyanide for ferric $P450_{SPα}$.** $P450_{SPα}$ was dissolved in 100 mM potassium phosphate buffer (pH 7.0) containing 20% glycerol and a fatty acid. The fatty acid concentration was 100 μM (C_8–C_{13} fatty acids) or 50 μM (C_{14} fatty acid). *Ks* was determined from the spectral titration of $P450_{SPα}$ with potassium cyanide.

So-called substrate-induced spectral changes are often observed on the addition of a substrate or its analogues to ferric P450. However, no spectral change was detected on the addition of the fatty acids to ferric $P450_{SPα}$.

DISCUSSION

Monooxygenase P450s activate molecular oxygen and insert one oxygen atom into the hydrophobic parts of substrate molecules, such as the ω or ω-1 position of fatty acids. It seems, therefore, that P450s have hydrophobic heme environments, and P450-ligand interactions reflect individual differences in the heme environments, *i.e.* the structures of the active site of monooxygenase metabolizing hydrophobic compounds, among the P450s. On the other hand, peroxygenase $P450_{SPα}$ introduced an oxygen atom of hydrogen peroxide at the position adjacent to the carboxyl group of fatty acids. Most compounds which serve as external ligands of monooxygenase P450s could not gain access to the ferric heme iron of $P450_{SPα}$, while cyanide, an inorganic anion, could gain access to the ferric heme iron of $P450_{SPα}$, coordinating with it with higher affinity than in the cases of microsomal P450s, *i.e.* monooxygenases metabolizing hydrophobic compounds (Table I). Moreover, fatty acids with an optimal carbon chain length, substrates of $P450_{SPα}$, facilitated the binding of cyanide to the heme iron of $P450_{SPα}$, whereas the substrate of monooxygenase P450s in most cases interfered with the binding of external ligands to the heme iron (Tables I and II). Such unique features of the heme-ligand interactions of $P450_{SPα}$ may reflect the heme environment of this P450 and may be related to the function of this enzyme, *i.e.* as a peroxygenase catalyzing fatty acid hydroxylation at the α position. The consensus sequences found in the proximal and distal heme regions of the P450 superfamily were modified in $P450_{SPα}$. Similar modifications were found in P450s catalyzing peroxide-related reactions (*3*, *13–16*). Heme-ligand interactions have not been reported for these P450s, but their heme

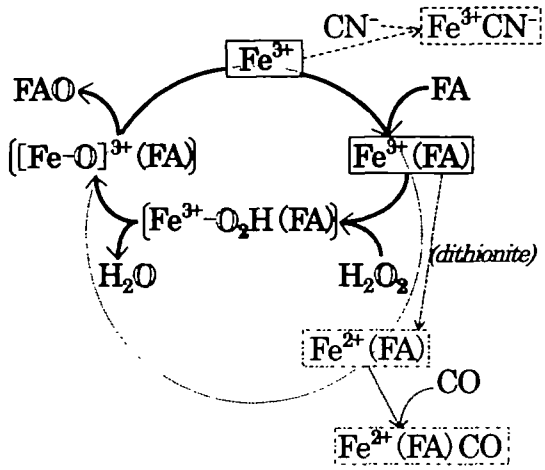

Fig. 4. **Proposed mechanism of the hydroxylation reaction catalyzed by peroxygenase P450$_{SPα}$.** FA and FAO represent the substrate, fatty acid, and the hydroxylated product, α-hydroxy fatty acid, respectively.

environments, which depend on the ternary structure of the heme pocket, may resemble that of P450$_{SPα}$. It is believed that the ternary structure of the heme pocket may be essentially conserved in all P450s, but differences in many details have been found among P450s *(9–12, 18, 19)*. The heme pocket of P450$_{SPα}$ may be relatively narrow for hydrophobic compounds, because only alkylamines (flexible molecules in shape) served as external ligands among nitrogen-containing compounds. The crystal structure of P450 catalyzing peroxide-related reactions has not yet been solved, and how the structure of P450$_{SPα}$ or a related P450 differs from those of monooxygenase P450s remains to be solved.

It is generally accepted that the P450 monooxygenase reaction proceeds as follows: (i) the substrate binds ferric P450 to form a ferric P450-substrate complex, (ii) this complex accepts one electron from NAD(P)H to give a ferrous P450-substrate complex, (iii) this complex combines with molecular oxygen to produce an oxygenated form, (iv) a second electron is introduced from NAD(P)H to the oxygenated form to yield "an unidentified intermediate", and (v) an "activated oxygen" is generated and inserted into the substrate to yield the oxygenated product. In this reaction mechanism, the introduction of the second electron to the oxygenated form of the ferrous P450-substrate complex (step 4) is believed to be the rate-limiting step of the overall reaction *(37, 38)*. Considering the analogy of this mechanism and that P450$_{SPα}$ uses an oxygen atom of hydrogen peroxide, but not of molecular oxygen, we can assume the mechanism of the peroxygenase reaction catalyzed by P450$_{SPα}$ shown in Fig. 4. As reported previously *(3, 6, 7)*, the peroxygenase reaction catalyzed by P450$_{SPα}$ is distinct from the nonspecific, non-enzymatic reactions of various hemoproteins or iron-containing compounds and hydrogen peroxide, and is related to the monooxygenase reactions of usual P450s. No spectral change was observed when hydrogen peroxide was added to ferric P450$_{SPα}$ at the concentration used for assaying the hydroxylation reaction in the absence of a fatty acid. Hydrogen peroxide did not affect the binding of cyanide to ferric P450$_{SPα}$. Therefore, the ferric P450$_{SPα}$–fatty acid complex may form first, and then this complex combines with hydrogen peroxide to produce the peroxy complex of P450$_{SPα}$, from which the activated oxygen is generated. Hydrogen peroxide can bind the ferric form of monooxygenase P450 to generate the oxygenated product *via* a peroxy compound in a "short circuit" path *(38)*. P450s catalyzing peroxide-related reactions also utilize peroxy compounds as substrates *(13–16)*, and we suppose that the peroxy complexes of these P450s are generated from the substrate complexes of the P450s. In the short circuit reaction, alkyl peroxides such as cumene hydroperoxide are more effective than hydrogen peroxide, while these alkyl peroxides are ineffective for the reaction of P450$_{SPα}$ *(7)*. In contrast with the short circuit reaction of monooxygenase P450, P450$_{SPα}$ exhibited a low K_m for hydrogen peroxide (72 μM) in the peroxygenase reaction *(7)*. Because the binding of substrates to ferric P450 proceeds very fast in the case of monooxygenase P450 *(37)*, it is reasonable to assume that the binding of hydrogen peroxide to a ferric P450$_{SPα}$-fatty acid complex is the rate-limiting step of the overall reaction. Thus, the hydroxylase reaction is sensitive to cyanide. On the other hand, the reaction is insensitive to CO, which binds ferrous P450, because the ferrous form is not involved in the mechanism of the reaction of P450$_{SPα}$. Further studies are necessary to clarify the details of the reaction mechanism of P450$_{SPα}$ and related P450 enzymes.

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