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Further Characterization of Hydrogen Peroxide-Dependent Fatty Acid α-Hydroxylase from *Sphingomonas paucimobilis*[1]

Isamu Matsunaga,*,[2] Maki Yamada,*,† Emi Kusunose,* Tokuhiko Miki,† and Kosuke Ichihara*

**Section of Molecular Regulation, Toneyama Institute for Tuberculosis Research, Osaka City University Medical School, 5-1-1 Toneyama, Toyonaka, Osaka 560-0045; and †Department of Ophthalmology, Osaka City University Medical School, 1-5-7 Asahi-machi, Abeno-ku, Osaka 545-8585*

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Although fatty acid α-hydroxylase (FAAH) activity has been detected in various species, FAAH has not been sufficiently characterized. In this report, we describe the properties of FAAH highly purified from *Sphingomonas paucimobilis*. The FAAH was purified by about 5,200-fold. Blotting analysis with a specific antibody against the FAAH showed that its apparent molecular mass was approximately 43 kDa. FAAH showed α-hydroxylation activity in the presence of H_2O_2, but little if any activity with cumene hydroperoxide, *t*-butyl hydroperoxide, or *t*-butyl peroxybenzonate. The K_m value for H_2O_2 was 72 μM. Highly purified FAAH oxidized various non-esterified saturated and unsaturated fatty acids including myristic acid, but not myristoyl-CoA. Potassium cyanide and sodium azide inhibited the FAAH activity in a concentration-dependent manner. Other respiratory chain inhibitors such as rotenone and antimycin A did not inhibit the activity. Among cytochrome P450 inhibitors, SKF-525A markedly inhibited the activity at the concentration of 2 mM, but CO did not. Imidazole, an inhibitor of plant α-oxidation, showed no inhibitory effect at 1 mM.

Key words: cytochrome P450, fatty acid, hydrogen peroxide, α-oxidation, *Sphingomonas paucimobilis*.

Fatty acid α-oxidation activity has been found in wide range of organisms. In plants, Castelfranco *et al.* (*1*) studied the α-oxidation system of straight-chain fatty acids such as palmitic acid using germinating peanut cotyledons. In this system, an H_2O_2-generating system involving an H_2O_2-forming oxidase such as glycolate oxidase was required for α-oxidation activity (*1, 2*). Hitchcock and James (*3*) investigated palmitic acid α-oxidation in pea leaves. They found 2-hydroxy fatty acids as intermediates of the α-oxidation. However, the enzymes catalyzing α-oxidation in these studies have not been sufficiently purified to allow their characterization in detail (*4*).

In mammals, Tsai *et al.* (*5*) demonstrated that phytanic acid (3,7,11,15-tetramethyl hexadecanoic acid) was α-oxidized by rat liver mitochondria. In this study, 2-hydroxy fatty acids were also identified as intermediates in the α-oxidation pathway. Thus, non-esterified fatty acids were considered to be initially α-hydroxylated to form 2-hydroxy fatty acids, followed by decarboxylation to remove the carboxyl carbon as CO_2. Recently, Singh *et al.* (*6*) reported that human phytanic acid α-oxidation occurred in peroxisomes but not mitochondria. Their results supported the pathway in which non-esterified fatty acids are α-hydroxylated (*7*). However, Mihalik *et al.* (*8*) and Croes *et al.* (*9*) reported that acyl-CoAs were utilized as substrates in the α-hydroxylation step to form 2-hydroxy acyl-CoA. Subcellular localization, substrate specificity, and cofactor requirements for phytanic acid α-oxidation are unclear, as the results of various reports are inconsistent (*5-11*).

Hoshi and Kishimoto (*12*) observed that α-hydroxylation of very long chain fatty acids such as lignoceric acid in the rat brain is associated with sphingolipid metabolism. α-Hydroxylation of lignoceric acid of rat brain required a heat-labile factor and heat-stable factors (*13, 14*). Although these factors were characterized to some extent, lignoceric acid α-hydroxylase itself has not been characterized, and the roles of heat-labile and heat-stable factors in the α-hydroxylation are unknown.

To address the above questions, purification of the enzymes catalyzing α-oxidation is necessary. If α-hydroxylation and the subsequent decarboxylation are catalyzed by distinct enzymes, their reaction rates should be measured separately. By measuring the α-hydroxylation activity, therefore, we partially purified fatty acid α-hydroxylase (FAAH) (*15*) from *Sphingomonas paucimobilis*, a bacterium with large amounts of sphingoglycolipid consisting of 2-hydroxy myristic acid (*16*). By use of the partially purified FAAH, we obtained evidence that hydrogen peroxide is directly involved in the bacterial α-hydroxylation of

[1] Sequence data of FAAH from *S. paucimobilis* has been entered in the DDBJ/EMBL/GenBank database under the accession number AB006957.

[2] To whom correspondence should be addressed. Tel/Fax: +81-6-853-5837, E-mail: matsunagai@msic.med.osaka-cu.ac.jp

Abbreviations: AS, ammonium sulfate; FAAH, fatty acid α-hydroxylase; P450, cytochrome P450.

fatty acid (*17*). Recently, we cloned the FAAH gene from this bacterium by expression cloning (*18*). Sequence analysis of this gene revealed that the FAAH was a novel member of cytochrome P450 (P450). In this study, we further purified native FAAH from *S. paucimobilis* to estimate whether native FAAH was identical to that gene product and characterized its catalytic properties. Similarities and differences between the enzymatic properties of the bacterial FAAH and the α-oxidation enzymes of other species are discussed.

MATERIALS AND METHODS

Materials—*Sphingomonas paucimobilis* EY2395^T was provided by Dr. Eiko Yabuuchi, Osaka City University Medical School. Fatty acids, cholic acid, DTT, hydroxylapatite, sodium azide, rotenone, and 8-hydroxyquinoline were purchased from Nacalai Tesque (Kyoto). Hydrogen peroxide, streptomycin sulfate, and ethylene glycol were purchased from Wako Pure Chemicals (Osaka). Myristoyl-CoA, antimycin A, and Reactive green 19 were obtained from Sigma Chemical (St. Louis, Missouri, USA). 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Chemical (Tokyo).

Assay for Fatty Acid α-Hydroxylation Activity—The standard method for assaying α-hydroxylation activity was described previously (*17*). Briefly, reaction mixtures contained 0.1 M Tris-HCl (pH 8.0), 0.2 mM H_2O_2, 60 μM fatty acid substrate, and enzyme preparation in a total volume of 0.2 ml. Incubation was performed at 37°C, and the reaction was terminated by addition of 20 μl of 2 N HCl. The amount of the enzyme preparation and the incubation period are indicated in tables and figure legends. When myristoyl-CoA was used as a substrate, 150 μl of 20% KOH/methanol was added to the mixture after incubation. The mixture was treated at 80°C for 20 min, then 150 μl of 5 N HCl was added. The substrate and the product were extracted with ethyl acetate and treated with ADAM. The ADAM-derivatized fatty acids were analyzed by HPLC according to a minor modification of the method described by Sawamura *et al.* (*19*). The amount of protein was determined by the method of Bradford (*20*).

Purification of Fatty Acid α-Hydroxylase—Bacterial cells were sonicated in 0.1 M Tris-HCl buffer (pH 7.6), and the soluble extract (crude extract) was obtained by ultracentrifugation. The extract was treated with streptomycin sulfate, the mixture was centrifuged, and the precipitate was discarded. The supernatant was fractionated with ammonium sulfate (AS) to obtain a 33-45% AS fraction. The AS fraction was dialyzed against 0.15 M potassium phosphate buffer (pH 7.0), then applied to a hydroxylapatite column equilibrated with the same buffer. After washing with an excess of the same buffer, the column was eluted with 0.45 M potassium phosphate buffer (pH 7.0). The eluate was concentrated and centrifuged at 100,000 × *g* for 60 min to remove insoluble materials. The supernatant was diluted with 10 volumes of 0.2 M potassium phosphate buffer (pH 7.0) containing 20% ethylene glycol, 0.2% cholic acid, and 1 mM DTT (buffer A), then applied to Reactive green 19 column equilibrated with buffer A. The column was washed with buffer A, followed by elution with buffer A containing 0.5 M KCl. The eluate was concentrated, then diluted with 10 volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 20% ethylene glycol, 0.4% cholic acid, 1 mM DTT, and 0.75 M AS (buffer B). This preparation was applied to a Phenyl-5PW-HPLC column (Bio-Rad, Richmond, California, USA), fractionated by HPLC with a linear gradient of 0.75 M to 0 M AS, and the FAAH fraction was collected. This fraction was concentrated, then diluted with 10 volumes of 0.1 M sodium phosphate buffer (pH 7.0) containing 30% ethylene glycol, 0.4% cholic acid, and 1 mM DTT (buffer C). The enzyme preparation was applied to a hydroxylapatite-KB-HPLC column (Koken, Tokyo) equilibrated with buffer C. After washing with buffer C, FAAH was fractionated with a linear gradient of 0.1 to 0.45 M sodium phosphate, and fractions of 0.5 ml were collected. Each fraction was analyzed by Western blotting using a specific antibody against recombinant FAAH.

Preparation of Specific Antibody against Recombinant Fatty Acid α-Hydroxylase and Western Blotting for Native FAAH—Expression and purification of the recombinant FAAH were described previously (*18*). A New Zealand White rabbit (female, 12 weeks old, SLC, Shizuoka) was immunized with the recombinant FAAH by the standard procedure (*21*). After immunization, serum was collected. The serum was fractionated twice with 50% AS. The AS fraction was dialyzed against phosphate-buffered saline (pH 7.4). Polyclonal IgG fraction was purified from the dialyzed AS fraction with an Ampure-PA kit (Pharmacia Biotech Inc., Piscataway, New Jersey, USA) according to the manufacturer's instruction manual. The IgG fraction was dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer D).

To obtain a specific antibody against the recombinant FAAH, we prepared a recombinant FAAH-conjugated affinity column using a Hitrap NHS-activated (Pharmacia). All experiments for preparation of the specific antibody described below were performed at room temperature. One milligram of the purified recombinant FAAH dissolved in 1 ml of buffer D containing 15% ethylene glycol and 0.25% cholic acid was applied to a Hitrap NHS-activated column. The column was allowed to stand for 1 h, then treated sequentially with 0.5 M ethanolamine, 0.5 M NaCl (pH 8.3) and 0.1 M acetate, 0.5 M NaCl (pH 4.0). This treatment was repeated three times. More than 90% of the applied protein was trapped in the column. After the column had been equilibrated with buffer D, the IgG fraction was applied to the column and washed with an excess of buffer D. The specific antibody against FAAH was eluted with 0.2 M sodium acetate buffer (pH 3.0).

We performed SDS-PAGE of each fraction separated with the hydroxylapatite-KB-HPLC column described above. Western blotting using the specific antibody was performed as described previously (*22*).

RESULTS

Purification and Identification of the Native FAAH—In this study, FAAH was purified 5,193-fold, and 9% of the FAAH activity was recovered (Table I). The apparent total activity following AS fractionation was higher than that of the crude extract, perhaps because inhibitors of FAAH were removed by AS fractionation. Therefore, the actual yield of FAAH was less than 5%. This lower yield may have been due to insolubilization of FAAH, because recovery was

improved by use of the buffer containing cholic acid. Non-ionic detergents such as Triton X-100, Tween 20, and Emulgen 911 did not improve recovery.

To determine the molecular mass of the native FAAH, we performed Western blotting with a specific antibody against the recombinant FAAH made using the expression plasmid containing the FAAH gene described previously (*18*). The elution profile of fatty acid α-hydroxylase on HPLC with the hydroxylapatite-KB column is shown in Fig. 1A. A single band, the amount of which paralleled α-hydroxylation activity, was detected by Western blotting in each fraction (Fig. 1B). The apparent molecular mass of the native FAAH on SDS-PAGE was approximately 43 kDa. A band corresponding to that detected by Western blotting was also observed on a silver-stained SDS-polyacrylamide gel (data not shown). However, a few other bands were also observed on the gel. The purified FAAH fraction did not show any significant decarboxylation activity when [1-^{14}C]-myristic acid was used as a substrate.

TABLE I. **Purification of fatty acid α-hydroxylase from *S. paucimobilis*.**

Step	Total protein (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Yield (%)	Purification (-fold)
Crude extract	1,450	0.15[a]	217.5	100	1
As fractionation	325.6	1.17[b]	381.0	175	8
Hydroxylapatite	11.0	18.8[c]	206.8	95	125
Reactive green 19	1.16	70.5[d]	81.8	38	470
Phenyl-5PW-HPLC	0.26	221[e]	57.5	26	1,473
Hydroxylapatite-KB-HPLC	0.026	779[f]	20.3	9	5,193

α-Hydroxylation activity was determined as described in "MATERIALS AND METHODS." The amount of enzyme preparation (protein) added to the reaction mixture and the incubation period in each purification step were as follows: [a]150 μg, 20 min; [b]80 μg, 20 min; [c]15 μg, 10 min; [d]5 μg, 10 min; [e]2 μg, 10 min; [f]0.2 μg, 10 min.

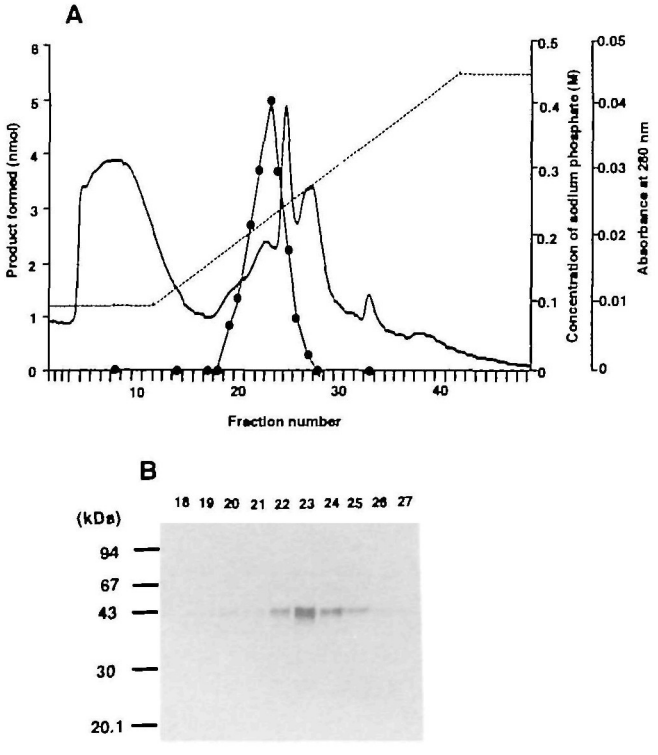

Fig. 1. **Elution profile of fatty acid α-hydroxylase on HPLC with hydroxylapatite-KB column (A) and Western blotting with the specific antibody (B).** (A) α-Hydroxylation activity of each fraction is indicated as the product formed in 10 min by 50 μl of each fraction. Closed circles indicate α-hydroxylation activities. The dotted line indicates concentration of sodium phosphate. The concentration of protein was monitored by absorption at 280 nm. (B) Western blotting was performed for fractions 18-27 as described in "MATERIALS AND METHODS."

Requirement of H_2O_2 and Substrate Specificity—FAAH essentially required H_2O_2 for its activity (Table II). Hydrogen peroxide-dependent α-hydroxylation activity increased in a concentration-dependent manner to reach almost maximum activity at the concentration of 200 μM. The K_m value for H_2O_2 was 72 μM (Fig. 2). In contrast to H_2O_2, addition of cumene hydroperoxide, *t*-butyl hydroperoxide, or *t*-butyl peroxybenzonate to the reaction mixture resulted in no or markedly reduced α-hydroxylation activity (Table II).

Next, we investigated the substrate specificity of FAAH in the presence of H_2O_2. Among non-esterified saturated fatty acids, myristic acid was most efficiently oxidized by FAAH (Table III). Longer-chain fatty acids, pentadecanoic acid to stearic acid, were also well utilized by FAAH. Non-esterified monounsaturated fatty acids, oleic acid and

TABLE II. **Effect of hydroperoxide on α-hydroxylation of myristic acid.**

Addition	Concentration (mM)	Specific activity (nmol/min/mg)
None	—	0.00
H_2O_2	0.2	46.8
Cumene hydroperoxide	0.2	0.00
	1	0.10
t-Butyl hydroperoxide	0.2	0.00
	1	0.49
t-Butyl peroxybenzonate	0.2	0.18
	1	0.00

α-Hydroxylation activity was determined with 5 μg of the enzyme preparation from the Reactive green 19 column. Incubation period was 10 min. As a substrate, myristic acid was used at the concentration of 60 μM.

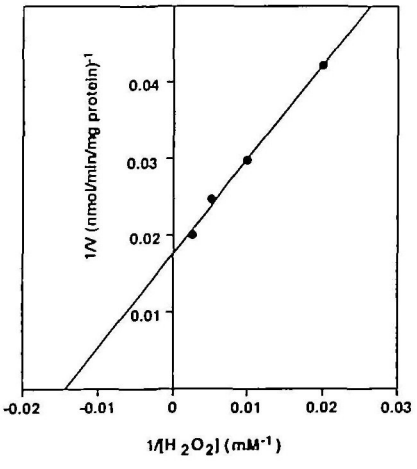

Fig. 2. **Lineweaver-Burk plot for H_2O_2.** The assay conditions was the same as in Table II, except the concentration of H_2O_2.

TABLE III. **Substrate specificity of fatty acid α-hydroxylase.**

Fatty acid substrate[a]	Expt. 1[b] Specific activity (nmol/min/mg)	Expt. 2[c] Specific activity (nmol/min/mg)
Non-esterified		
$C_{11:0}$	6.66	—
$C_{12:0}$	22.6	—
$C_{13:0}$	35.7	—
$C_{14:0}$	54.6	768
$C_{15:0}$	52.2	—
$C_{16:0}$	47.4	—
$C_{16:1}$	70.0	—
$C_{17:0}$	40.5	—
$C_{18:0}$	41.7	—
$C_{18:1}$	69.2	—
CoA derivative		
$C_{14:0}$	—	0

[a]The assays using non-esterified fatty acids or myristoyl CoA as substrates are described in "MATERIALS AND METHODS." The concentration of each substrate in the reaction mixture was 60 μM. [b]α-Hydroxylation activity was determined with 5 μg of the enzyme preparation from the Reactive green 19 column. Incubation period was 10 min. [c]α-Hydroxylation activity was determined with 0.2 μg of the enzyme eluted from the hydroxylapatite-HPLC column. Incubation period was 10 min.

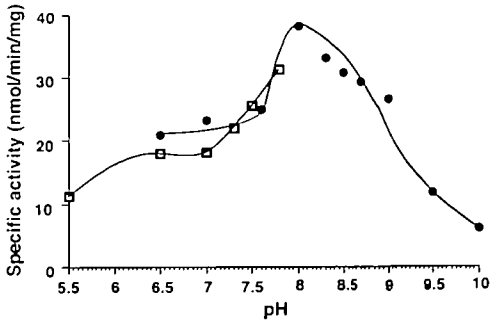

Fig. 3. **Effects of pH on myristic acid α-hydroxylation.** The assay conditions were the same as in Table II, except pH of the reaction mixture. Closed circles and open squares indicate α-hydroxylation activities with Tris-HCl buffer and potassium phosphate buffer, respectively.

TABLE IV. **Effects of various inhibitors on α-hydroxylation of myristic acid.**

Addition	Concentration	Relative activity[a] (%)
None	—	100
KCN	5 mM	14
NaN_3	5 mM	31
8-Hydroxyquinoline	1 mM	102
Rotenone	10 μM	101
Antimycin A	5 μg/ml	105
SKF-525 A	5 mM	7
$CO:O_2$[b]	(4:1)	104
Imidazole	1 mM	107

[a]The assay conditions were the same as in Table II, except for addition of inhibitors. Specific activity without the inhibitor was 37.7 nmol/min/mg protein. [b]The gas phase in the reaction tube was replaced by $CO:O_2$ (4:1).

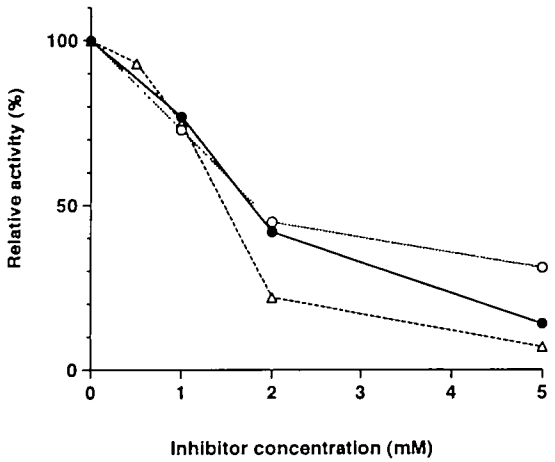

Fig. 4. **Inhibitory effect of potassium cyanide, sodium azide, and SKF-525A.** Closed circles, open circles, and open triangles indicate α-hydroxylation activities at various concentrations of potassium cyanide, sodium azide, and SKF-525A, respectively. The assay conditions and specific activity without inhibitors were the same as in Table IV.

palmitoleic acid, were oxidized more efficiently than myristic acid. When the enzyme preparation prior to hydroxylapatite-KB-HPLC was used, acyl-CoAs were oxidized similarly to or a little less than non-esterified fatty acids, to form the corresponding non-esterified 2-hydroxy fatty acids (data not shown). However, we also found that this enzyme preparation had acyl-CoA hydrolase activity which decreased accompanying purification of FAAH (data not shown). Finally, the enzyme prepared by hydroxylapatite-KB-HPLC showed no acyl-CoA hydrolase activity. Thus, we used this final enzyme preparation to examine whether the FAAH utilized acyl-CoA as a substrate. We found that FAAH did not oxidize myristoyl-CoA (Table III).

Effects of pH and Various Inhibitors—Subsequently, effects of pH and various inhibitors of respiratory chain and P450 were investigated. Significant α-hydroxylation activity was observed over a wide range of pH (Fig. 3). Maximum activity of FAAH was detected at pH 8.0. Among the respiratory chain inhibitors tested, KCN and NaN_3 strongly inhibited the activity in a concentration-dependent manner (Table IV, Fig. 4). The inhibition ratios of KCN and NaN_3 were almost the same at concentrations of 2 mM or less, but at 5 mM, KCN inhibited the activity more strongly than NaN_3 did. 8-Hydroxyquinoline, rotenone, and antimycin A did not show significant inhibitory effects on α-hydroxylation activity (Table IV). Watkins *et al.* (*23*) showed that the α-oxidation activity of phytanic acid was inhibited by the addition of rotenone and antimycin A. However, a combination of rotenone and antimycin A did not affect bacterial FAAH activity (data not shown). Among the P450 inhibitors tested, SKF-525A markedly inhibited α-hydroxylation activity when the FAAH fraction separated by Reactive green 19 column chromatography was used as an enzyme preparation. However, SKF-525A showed no significant inhibitory effect when the enzyme preparation prior to Reactive green 19 column fractionation was used. CO did not inhibit the activity. Imidazole did not inhibit α-hydroxylation activity at the concentration of 1 mM.

DISCUSSION

Despite efforts to clarify the properties of fatty acid α-oxidation enzyme(s) in various species, purification of FAAH has not been successful. In most previous studies of fatty acid α-oxidation, cell homogenate or organelles were used as enzyme preparations (*5, 8-12, 24*). However, to clarify the enzymatic properties of FAAH precisely, purification of FAAH is necessary. Thus, in this study, we highly purified the FAAH from *S. paucimobilis*.

The FAAH fraction purified from *S. paucimobilis* showed no decarboxylation activity, indicating that this enzyme catalyzes only α-hydroxylation. Borge *et al.* (*25*) reported that α-oxidation was catalyzed by only one enzyme, which was purified from cucumber by measuring CO_2 release. If so, this plant enzyme may be distinct from the bacterial hydroxylase.

Recently, we cloned the FAAH gene from *S. paucimobilis* (*18*). To evaluate whether native FAAH was identical to this gene product, we carried out further purification of the FAAH from *S. paucimobilis* (Table I) and identified FAAH by SDS-PAGE (Fig. 1). The molecular weight of the native FAAH was almost the same as that calculated from the predicted amino acid sequence of the FAAH gene product. These results suggested that the purified native FAAH was identical or at least very similar to the cloned gene product, and thus this P450 enzyme is actually functional as FAAH in this bacterium.

The bacterial FAAH could be saturated with small amounts of H_2O_2, and its hydroperoxide requirement was specific for H_2O_2 (Table II). Although some P450s utilize H_2O_2, much larger amounts of H_2O_2 are usually required for optimal activity (*26*). It is unclear why the FAAH is efficiently saturated with such small amounts of H_2O_2. Interestingly, we recently found structural similarity of amino acid sequence between FAAH and peroxide substrate-metabolizing plant P450s by alignment analysis (*18*). Thus, further studies including site-directed mutagenesis are necessary to clarify the catalytic mechanism of H_2O_2-dependent FAAH. In other species, the involvement of H_2O_2 in α-oxidation is not necessarily clear. In mammals, an H_2O_2-generating system generally inhibited the α-oxidation activity, and catalase did not affect this activity (*5, 12, 27*). In contrast, H_2O_2 or an H_2O_2-generating system showed various effects on plant α-oxidation. An H_2O_2-generating system was potentially required for α-oxidation activities of peanut and pea leaf (*28*), whereas in cucumber, H_2O_2 showed inhibitory effects (*29*). However, catalase inhibited the α-oxidation activity of cucumber, suggesting the involvement of H_2O_2.

The bacterial FAAH oxidized a wide range of nonesterified fatty acids, whereas the highly purified FAAH did not oxidize myristoyl-CoA (Table III). Mihalik *et al.* (*8*) reported that the hydroxylase of the phytanic acid α-oxidation pathway utilized phytanoyl-CoA as a substrate, but not phytanic acid. Thus, they named this enzyme "phytanoyl-CoA α-hydroxylase." Mihalik *et al.* (*30*) and Jansen *et al.* (*31*) reported mouse and human phytanoyl-CoA α-hydroxylase genes. These genes did not show any significant homology to previously reported genes that encode proteins of known function, including P450s.

α-Hydroxylation activity of bacterial FAAH was inhibited by KCN and NaN_3, but not 8-hydroxyquinoline, rotenone, or antimycin A (Table IV). Cyanide and azide are heme-ligands but do not generally act as P450 inhibitors. Therefore, sensitivity of FAAH to KCN and NaN_3 was unusual, although aromatase, a P450, is reported to be sensitive to KCN and NaN_3 (*32, 33*). Inhibition by cyanide and azide was reported in rat brain α-hydroxylation (*12, 24*). In rat brain, Shigematsu and Kishimoto (*24*) demonstrated that CO and SKF-525A did not show any inhibitory effects. However, insensitivity to CO and SKF-525A does not exclude the possibility that P450 is involved in α-hydroxylation, because aromatase was reported not to be inhibited by CO (*32, 33*) and other P450 inhibitors, including SKF-525A, do not always inhibit all P450s. In addition, it was reported that H_2O_2-supported hydroxylation by a P450 enzyme was inhibited by cyanide but not CO (*26*). In fact, H_2O_2-dependent α-hydroxylation by bacterial FAAH, a P450 enzyme, was not inhibited by CO. Imidazole was reported to show a strong inhibitory effect on α-oxidation of plants, including peanut cotyledon, pea leaf, and cucumber, at the concentration of 1 mM or less (*1, 3, 29*). Bacterial FAAH differed from these plant enzymes in its sensitivity to imidazole.

Here, we have described some properties of the FAAH from *Sphingomonas paucimobilis*. This bacterial FAAH showed unique properties as a P450: requirement of H_2O_2, sensitivity to KCN and NaN_3, and insensitivity to CO. In other species, therefore, it should be carefully determined whether a P450-like enzyme is involved in α-oxidation. Higher purification and molecular cloning of FAAH are suitable means to resolve this question.

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