Molecular Cloning and Expression of Fatty Acid α-Hydroxylase from *Sphingomonas paucimobilis*  

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Fatty acid α-hydroxylase (FAAH) catalyzes the initial reaction in α-oxidation of fatty acid to produce 2-hydroxy fatty acid. FAAH activity has been detected in a wide range of organisms from prokaryotes to eukaryotes. Here, we describe cloning of the FAAH gene from *Sphingomonas paucimobilis*, a spingolipid- and 2-hydroxymyristic acid-rich bacterium. The isolated gene encoded 415 amino acids. A homology search revealed that amino acid sequences highly conserved in cytochrome P450 (P450) were present in FAAH. Although the heme-binding cysteine was recognizable at position 361, the consensus in the heme-binding region was modified by an insertion. Overall, FAAH has no significant identity to the known P450s. CO difference spectrum of recombinant FAAH showed the characteristic one of P450, except this peak was at 445 nm. These results suggest bacterial FAAH is a novel member of the P450 superfamily.

α-Oxidation of fatty acids is widely observed in bacteria (1), yeasts (2), plants (3, 4), and mammals (5, 6). This reaction pathway results in catalysis of fatty acid to produce the corresponding fatty acid with one less carbon atom, where the fatty acid is first hydroxylated at the 2-position and sequentially decarboxylated to release carbon dioxide. This first and rate-limiting reaction is catalyzed by fatty acid α-hydroxylase (FAAH). In mammals, the α-oxidation pathway is essential for catabolism of 3-methyl branched fatty acids such as phytanic acid in dietary sources like ruminant fats. Refsum disease is a human inherited disorder resulting from FAAH deficiency, in which phytanic acid exclusively accumulates in the patients’ organs such as the liver and in their serum (7). In the mammalian brain, α-hydroxylation activity of very long chain fatty acids such as lignoceric acid has been reported to be associated with brain development, where 2-hydroxy fatty acid thus produced is introduced into cerebroside utilized for myelin formation (8, 9). Using organelles isolated from liver or brain, the α-oxidation pathway has been investigated. However, the enzymatic properties of FAAH described in several reports were inconsistent, e.g. cofactor requirement or substrate specificity (5, 6, 10–15). On the other hand, in plants, characterization of FAAH has been more successful. It is noted that a plant FAAH partially purified from cotyledons of germinating peanut seeds requires an H$_2$O$_2$-generating system consisting of glycolate oxidase for its activity (3, 16, 17).

We have investigated FAAH isolated from *Sphingomonas paucimobilis*, a bacterium containing large amounts of sphingoglycolipids (18). Most of these sphingoglycolipids contain 2-hydroxymyristic acid (19). Our previous studies indicated that FAAH from *S. paucimobilis* required H$_2$O$_2$ for its activity and the oxygen atom of the H$_2$O$_2$ was introduced into myristic acid to produce 2-hydroxymyristic acid (20). We attempted to purify the FAAH. However, complete purification of FAAH was not successful, perhaps because the amount of FAAH of *S. paucimobilis* was very small and its activity was too labile. Thus, we decided to clone the FAAH gene from this bacterium. Here, we describe cloning and expression of the FAAH gene from *S. paucimobilis*, and the characterization of the recombinant FAAH.

**MATERIALS AND METHODS**

*Bacteria and Plasmids—* *S. paucimobilis* EY2395$^T$ (19) was gifted from Dr. Eiko Yabuuchi, Osaka City University Medical School, Osaka, Japan. *Escherichia coli* JM109 and *E. coli* BL21 were purchased from Toyobo Co., Ltd. (Osaka, Japan). Plasmids pUC18 and gEM-4T-3 were obtained from Takara Shuzo Co., Ltd. (Shiga, Japan) and Pharmacia Biotech Inc., respectively.

Isolation and Sequencing of Genomic DNA Clones Encoding FAAH—General cloning techniques were carried out essentially as described by Davis et al. (21). Genomic DNA from *S. paucimobilis* EY2395$^T$ was partially digested by Sou3AI. Three- to 10-kb fragments were ligated into BamHI-digested pUC18, and these constructs were transfected into *E. coli* JM109. The transformed cells were cultured in Luria broth (LB) containing ampicillin. At log phase of cell growth, isopropyl-1-thio-β-D-galactopyranoside (IPTG, Wako Pure Chemical Co., Osaka, Japan) was added into the culture medium to a final concentration of 1 mM, and the cells were cultivated for another 4 h. The cultivated cells were collected by centrifugation and disrupted by sonication in an appropriate volume of 0.1 M Tris-HCl buffer (pH 7.5). After centrifugation to remove disrupted cells, the α-hydroxylation activity in the resultant supernatant was assayed. For rescreening to obtain the DNA fragment encoding the N terminus of the FAAH, genomic DNA from *S. paucimobilis* was digested by HinClII. The digested DNAs were size-selected, and 1.5–2.5-kb DNA fragments were ligated into HinClII-digested pUC18, after which colony hybridization was performed. Labeling of the probe and detection of hybridized fragments were performed with the digoxigenin DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany).

Sequencing of DNA fragments was performed using the dye-terminator cycle sequencing kit and an ABI 373A DNA sequencer (PerkinElmer, Foster City, CA). Alignment analysis was performed as described by Gotoh (22).

Overexpression, Purification, and Characterization of FAAH Protein—An EcoRI blunt-ended XhoI fragment containing the FAAH gene was ligated into EcoRI, Smal-digested pGEM-4T-3. The construct was consistent.
transfected into *E. coli* BL21, and transformed cells were cultured in LB containing ampicillin at 25 °C. At log phase, IPTG was added to the culture medium to a final concentration of 0.1 mM. After another 20 h of cultivation, cells were collected by centrifugation and disrupted by sonication in 0.1M Tris-HCl buffer (pH, 7.5), 20% ethylene glycol, 1 mM dithiothreitol (buffer A) containing 1% cholic acid, 0.1% SDS, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (Nacalai Tesque, Inc., Kyoto, Japan). The supernatant obtained by centrifugation at 100,000 *g* for 60 min was diluted by addition of 2 volumes of buffer A. Glutathione S-transferase (GST)-FAAH fusion protein was bound to glutathione-Sepharose (Pharmacia) and then eluted with buffer A containing 0.3% cholic acid, 0.05% SDS, 1.5 M urea, and 5 mM reduced glutathione. The eluate was treated with thrombin at 25 °C for 20 h. The resultant eluate was dialyzed against 0.1 mM sodium phosphate buffer (pH 7.0), 0.2 mM H~2~O~2~, 60 mM myristic acid, and 0.5 mM of purified FAAH in a total volume of 0.2 ml. Incubation was performed at 37 °C for 10 min. The reaction was terminated by addition of 2N HCl. The substrate and

FIG. 1. Schematic representation of two genomic DNA fragments containing part of the FAAH gene. Solid bars indicate *Sau3AI* fragment and *HincII* fragment. The arrow shows the FAAH gene. Open bars indicate *pOC4* insert (*OC4*) and its deletion mutants.

FIG. 2. Nucleotide sequence of 1.3-kb fragment containing the FAAH gene and deduced amino acid sequence of FAAH (A) and alignment of the amino acid sequence of helix-K (B), aromatic region (C), and heme-binding region (D). A, the deduced amino acid sequence is represented under the nucleotide sequence by single-letter code. The asterisk and double underline indicate the termination codon and putative Shine-Dalgarno sequence, respectively. Helix-I, helix-K, aromatic region, and heme-binding region are underlined. Heme-binding cysteine is boxed. B–D, residues identical with those of FAAH are shaded, and heme-binding cysteines are indicated as bold letters. *Hum. CYP 3A5*, human thromboxane synthase; *Hum. CYP 5A1*, human prostacyclin synthase; *Flax. CYP 74A1*, flaxseed allene oxide synthase; *B. pp CYP 74B1*, bell pepper fatty acid hydroperoxide lyase.

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product were extracted with ethyl acetate and treated with 9-anthryl-diazomethane (Funakoshi Chemical Co., Tokyo, Japan). The 9-anthryl-diazomethane-derivatized fatty acids were analyzed by HPLC as described previously (24).

RESULTS AND DISCUSSION

For cloning of the FAAH gene, a genomic library was constructed using pUC18 and E. coli JM109 transfected with the constructed plasmids was cultivated. After induction by IPTG, the cells were collected, disrupted by sonication, and centrifuged, and then the α-hydroxylation activity in the resultant supernatant was assayed. Four thousand clones were screened, and one positive clone was obtained. The plasmid designated pOC4 isolated from this clone contained a 3.3-kb insert (OC4) (Fig. 1). Sequence analysis of this insert revealed two significant open reading frames (ORFs) at 5′- and 3′-ends. We found that the coding sequence of lacZ of the vector plasmid was connected in frame to 5′-ORF of OC4. Deletion mutants at the 5′-end or 2.8 kb from the 3′-end abolished α-hydroxylation activities. Therefore, we concluded that the 5′-ORF encoded the FAAH. Surprisingly, a homology search for the deduced amino acid sequence of the 5′-ORF revealed homology to sequences conserved in cytochrome P450 (P450); in particular, a highly homologous sequence to the aromatic region of P450 (25) was found in FAAH (Fig. 2). Alignment analysis for FAAH and the known P450s suggested that the N-terminal of FAAH was missing, and thus we screened the library constructed with HindII-digested genomic DNA using a 513-bp fragment from the 5′-end of OC4 (from Sau3AI at 5′-end of OC4 to HindII (SalII)) as a probe (Fig. 1). We obtained a 2.4-kb fragment and found the initiation codon at 54 bp upstream from the 5′-end of OC4. A termination codon was located in frame 51 bp upstream from the initiation codon (Fig. 2A). The sequence from −29 to −24, AAGGAG, matched to the Shine-Dalgarno consensus sequence, although it was so far from the initiation codon. Consequently, the FAAH gene encoded a protein of 415 amino acids and the calculated molecular weight was 46,485. As mentioned above, significant homology to the highly conserved regions among the P450 superfamily was observed. Particularly, the sequence of the aromatic region of FAAH was highly homologous to that of P450: 75% identical to that of CYP 3A5 (26) (Fig. 2C). With the exception of two cases, Bacillus megaterium CYP 102A and Anaobena sp. ORF3-encoded sequence, bacterial P450s that belong to the B-class generally lack the aromatic region, while all mammalian P450s that belong to the E-class have the aromatic region (25). Therefore, the bacterial FAAH reported here constitutes a third case belonging to the E-class. In helix-K, the EXX motif, which is completely conserved in the P450 superfamily, was found in FAAH (Fig. 2B). Although the heme-binding cysteine was found at position 361, the consensus motif of the heme-binding region (GXXGXX), was modified, where conserved phenylalanine was substituted to glutamine and seven amino acids were found between conserved glycine and cysteine residues (Fig. 2D). Such insertions were also found in plant P450s, CYP 74A1 (27) and CYP 74B1 (28), in which the phenylalanine was inserted into the pGEM-4T-3 expression vector to construct a GST-FAAH fusion gene. In this construct, FAAH was truncated by 18 amino acids at N terminus, but contained all the elements necessary for its activity. The expressed fusion protein was isolated with glutathione-Sepharose and digested with thrombin, and then the FAAH was purified by HPLC with a hydroxylapatite column (Fig. 4B). The purified FAAH showed myristic acid α-hydroxylation activity in the presence of H₂O₂ to produce 2-hydroxymyristic acid, while no such activity was detected in the absence of H₂O₂ (Fig. 4C). The Km value for H₂O₂ was approximately 60 μM, which was similar to that of landin H₂, and also do not require molecular oxygen, have substitution of threonine at the appropriate position in helix-I, which is believed to be the critical residue for O₂ activation (31). The sequence of helix-I of FAAH showed no significant homology to those of other P450s and also lacked an adjacent threonine residue. These findings also supported the hypothesis that FAAH utilizes H₂O₂ but dose not require O₂ activation by reductase such as NADPH-cytochrome P450 reductase (20). Despite the high degree of conservation of sequences found in P450s, overall FAAH had no significant identity to the known P450s (less than 25% identity). However, the hydrophobicity profile (32) of FAAH was similar to those of other bacterial P450s, P450cam (CYP 101A) and P450BM3 (CYP 102A) hydroxylase domain, which are soluble enzymes (33) (Fig. 3), supporting the observation that FAAH was isolated in the soluble fraction (18).

To examine whether the FAAH gene-encoded protein has the properties of P450, we purified the FAAH overexpressed in E. coli. As shown in Fig. 4A, the EcoRI-XbaI fragment of pOC4 was inserted into the pGEM-4T-3 expression vector to construct a GST-FAAH fusion gene. In this construct, FAAH was truncated by 18 amino acids at N terminus, but contained all the elements necessary for its activity. The expressed fusion protein was isolated with glutathione-Sepharose and digested with thrombin, and then the FAAH was purified by HPLC with a hydroxylapatite column (Fig. 4B). The purified FAAH showed myristic acid α-hydroxylation activity in the presence of H₂O₂ to produce 2-hydroxymyristic acid, while no such activity was detected in the absence of H₂O₂ (Fig. 4C). The Km value for H₂O₂ was approximately 60 μM, which was similar to that of landin H₂, and also do not require molecular oxygen, have substitution of threonine at the appropriate position in helix-I, which is believed to be the critical residue for O₂ activation (31). The sequence of helix-I of FAAH showed no significant homology to those of other P450s and also lacked an adjacent threonine residue. These findings also supported the hypothesis that FAAH utilizes H₂O₂ but dose not require O₂ activation by reductase such as NADPH-cytochrome P450 reductase (20). Despite the high degree of conservation of sequences found in P450s, overall FAAH had no significant identity to the known P450s (less than 25% identity). However, the hydrophobicity profile (32) of FAAH was similar to those of other bacterial P450s, P450cam (CYP 101A) and P450BM3 (CYP 102A) hydroxylase domain, which are soluble enzymes (33) (Fig. 3), supporting the observation that FAAH was isolated in the soluble fraction (18).

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native FAAH partially purified from S. paucimobilis. Other peroxo compounds, cumene hydroperoxide, \(t\)-butyl hydroperoxide, and \(t\)-butyl peroxybenzonate, were not effective (data not shown). We observed that, like the recombinant FAAH, native FAAH was specific for H\(_2\)O\(_2\). The turnover rate of myristic acid \(\alpha\)-hydroxylation by FAAH was very high, comparable to that of P450BM3, which efficiently catalyzes lauric acid (\(\omega\)-2)-hydroxylation (34); specific activity of FAAH was 838 nmol/min/nmol of protein. We next examined the spectral properties of the FAAH. The CO difference spectrum of FAAH showed the characteristic one of P450, except this peak was at 445 nm (Fig. 4D).

On the basis of the results of sequence and spectral analyses of FAAH, we concluded that bacterial FAAH was a novel member of the cytochrome P450 superfamily. Previously, Muralidharan and Kishimoto (14) showed partial inhibition of phytanic acid \(\alpha\)-oxidation of rat liver by CO, indicating a possible involvement of P450. Recently, Pahan et al. (35) reported that CO inhibited phytanic acid \(\alpha\)-hydroxylation activity of peroxisomes from human liver. Despite the analysis of CO difference spectrum of the bacterial FAAH revealed that...
Fe(II)-CO complex was formed (Fig. 4D), the inhibition of the hydroxylation activity by CO, as can be seen in most of P450s, was not observed (data not shown).

Most recently, Borge et al. (36) reported that palmitic acid can be α-oxidized to release CO₂ by only one enzyme purified from cucumber, although its purification was not complete, judging from the observation on SDS-polyacrylamide gel electrophoresis. If fatty acids were first α-hydroxylated, and then decarboxylated in α-oxidation, this enzyme must catalyze these two reactions. FAAH described in this report, however, did not show significant decarboxylase activity. Therefore, at least in bacteria, decarboxylase and FAAH could exist separately. Furthermore, there seems to be a resemblance between the bacterial FAAH and a plant FAAH (16, 17). Both enzymes catalyze α-hydroxylation, at least in some species of plants. In any case, further studies are necessary to clarify these points.

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