Long-chain fatty acids can be metabolized to \( C_{n-1} \) aldehydes by \( \alpha \)-oxidation in plants. The reaction mechanism of the enzyme has not been elucidated. In this study, a complete nucleotide sequence of fatty acid \( \alpha \)-oxygenase gene in rice plants (Oryza sativa) was isolated. The deduced amino acid sequence showed some similarity with those of mammalian prostaglandin H synthases (PGHSs). The gene was expressed in Escherichia coli and purified to apparently homogenous state. It showed the highest activity with linoleic acid and predominantly formed 2-hydroperoxide of the fatty acid (\( C_\text{n} \)), which is then spontaneously decarboxylated to form corresponding \( C_{n-1} \) aldehyde. With linoleic or linolenic acids as a substrate, rice \( \alpha \)-oxygenase formed no product having a \( \lambda_{\text{max}} \) at approximately 234 nm, which indicated that the enzyme could not oxygenize the pentadiene system in the substrate. The spectroscopic feature of the purified enzyme in its ferrous state is similar to that of mammalian PGHSs, whereas that of dithionite-reduced state showed significant difference. Site-directed mutagenesis revealed that His-158, Tyr-380, and Ser-558 were essential for the initial step of \( \alpha \)-oxidation reaction and a residue involved in substrate binding, respectively. This finding suggested that the initial step of the oxygenation reaction in \( \alpha \)-oxygenase has a high similarity with that of PGHSs. The rice \( \alpha \)-oxygenase activity was inhibited by imidazole but hardly inhibited by nonsteroidal anti-inflammatory drugs, such as aspirin, ibuprofen, and flurbiprofen, which are known as typical PGHS inhibitors. In addition, peroxidase activity could not be detected with \( \alpha \)-oxygenase when palmitic acid 2-hydroperoxide was used as a substrate. From these findings, the catalytic resemblance between \( \alpha \)-oxygenase and PGHS seems to be evident, although there still are differences in their substrate recognitions and peroxidation activities.

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Long-chain fatty acids can be metabolized by \( \alpha \), \( \beta \), and \( \omega \)-oxidation in both plant and animal tissues (1). The enzyme systems responsible for \( \beta \)-oxidation have been isolated and extensively studied. It was confirmed that the enzymes localize on the mitochondria and peroxisomes. Cytochrome P450s in the endoplasmic reticulum catalyze the fatty acid \( \omega \)-hydroxylation (2). There are at least three types of \( \alpha \)-oxidation systems. In mammals, \( \alpha \)-oxidation is essential for degradation of branched-chain fatty acids with a methyl group at the \( \beta \)-position, which prevents the initial step of \( \beta \)-oxidation. This reaction is carried out by phytanoyl-CoA hydroxylase, which needs 2-oxoglutarate as an essential cofactor (3). The mutation of a gene encoding this enzyme causes Refsum’s disease in human. Phytanoyl-CoA hydroxylase can also act on straight-chain fatty acids; however, this activity seems to be important only in brain and nerve tissues. In Sphingomonas paucimobilis, a spingolipid-rich and 2-hydroxymyristic acid-rich bacterium, the initial reaction of \( \alpha \)-oxidation is catalyzed by a novel type of cytochrome P450, fatty acid \( \omega \)-hydroxylase (4). Fatty acid \( \omega \)-hydroxylase requires hydrogen peroxide for the hydroxylation of myristic acid to produce 2-hydroxymyristic acid. In contrast, plant \( \alpha \)-oxidation systems are distinct from those in mammals or bacteria. Recently, a gene encoding pathogen-inducible oxygenase (PIOX) and its homolog have been isolated in Nicotiana tabacum and Arabidopsis thaliana (10). The protein derived from the gene was expressed in insect cells and found to cause uptake of molecular oxygen in the presence of polyunsaturated fatty acids such as linoleic, linolenic, and arachidonic acids. Later, the protein was identified as fatty acid \( \omega \)-oxygenase, which catalyzes the conversion of linoleic acid and the other fatty acids into the corresponding \( R \)-2-hydroperoxy fatty acids (11). Interestingly, the primary structures of plant \( \omega \)-oxygenases show similarity with those of mammalian PGHSs, although the substrates and products of these two oxygenases are quite different from each other. So far, there has been no report on the catalytic properties of plant \( \omega \)-oxygenases, and no one knows whether there exists some catalytic resemblance between plant \( \omega \)-oxygenases and mammalian PGHSs. By a BLAST search on the rice EST data base, we noticed that one
of the EST clones has high sequence similarity with tobacco PIOX. Fatty acid α-oxidation gene of the monocotyledonous plants has never been characterized. Thus, to elucidate the reaction mechanism of α-oxidation and the function of a given amino acid residue for the reaction, the fatty acid α-oxidation gene of rice plants (Oryza sativa) was cloned and expressed in Escherichia coli, and the properties of the recombinant enzyme were analyzed.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Condition—**Seeds of rice plants (O. sativa L. cv. Kimnara) were sterilized with 10% sodium hypochlorite solution for 1 day and then grown hydroponically in a chamber at 28 °C under 14-h light/10-h dark photoperiod.

**Expression and Purification of α-Oxidase—**EST clone S10043 from green shoots of rice (O. sativa L. cv. Nipponbare) was supplied by The Ministry of Agriculture, Forestry, and Fisheries DNA Bank (bank.dna.affrc.go.jp). For the expression in E. coli, SacI site was introduced just before the initiation codon of the cDNA by PCR, and then the full-length of the open reading frame digested with (nucleotide) was cloned and expressed in E. coli expression vector pQE-30 (Qiagen). The primers used for the PCR were 5′-ACGCGTCTATGAGGCTGGAGCCTCTTTACAAGGC-3′ (the initiation codon is underlined) as the sense primer and 5′-CCATATGCTCACTCCCATCC-CACC-3′ as the antisense primer. E. coli cells (strain M15) were transformed with the construct. An overnight culture (50 ml) of the transformant was inoculated into 500 ml of 2× YT medium supplemented with 100 μg/ml ampicillin and 30 μg/ml kanamycin. The cells were grown at 37 °C until the A600 reached 0.6–0.8. Cultures were chilled to 25 °C, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and then the cells were further cultured for 12 h at 25 °C. Bacterial cells expressing the recombinant protein were harvested and suspended in 10 ml of 20 mM sodium phosphate (pH 7.0) containing 300 mM NaCl. The cells were lysed by using 25–15 s pulses of sonication with a tip-type sonicator (UR-150P, Tomy Co., Tokyo, Japan).

The lysate was centrifuged at 4000 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min. The recombinant α-oxidase was solubilized with 8 ml/g wet weight of the cells of 20 mM sodium phosphate (pH 7.0) containing 300 mM NaCl and 0.1% Nonidet P-40 (buffer A) on ice for 45 min with stirring. The suspension was centrifuged at 100,000 × g for 60 min, and the supernatant was applied to a 1.6 × 1.5 cm TALON metal affinity column (CLONTECH Co.) equilibrated with buffer A (pH 7.0). The column was washed with 100 ml of the buffer A at pH 7.0. Proteins were eluted from the column in buffer B at pH 5.0. The eluted fractions having O2 uptake activity were pooled and immediately adjusted at pH 7.0.

**Enzyme Assay and Protein Content—**Oxygen consumption was measured with a Clark-type oxygen electrode (YSI 5331, Yellow Springs Instrument) at 25 °C in 100 ml Hepes buffer (pH 7.2) with 1.8 ml of the total volume. The reaction was started by the addition of the enzyme to the reaction mixture containing 0.55 mM linoleic acid and 0.002% Nonidet P-40. The activity (1 katal) was defined as the quantity of enzyme required to oxidize 1 μmol of O2/sec at 25 °C. The enzyme activity was measured by DNA sequencing.

**Fatty-acid Hydroperoxide—**Reaction mixture of 10 μl of palmitic acid solution (50 mM, suspended in 0.2% EtOH; final concentration, 500 μM), the enzyme solution corresponding to 0.95 μg of protein, and 100 μM Hepes buffer (pH 7.2) with 5 ml of the total volume were incubated on ice for 15 min. The hydroperoxides formed were converted into esters with diazomethane (ADAM) reagent. The detection of the resultant ADAM esters of hydroperoxides was performed with reversed-phase HPLC as described previously (5). To reveal stereocatalysis of 2-hydroperoxy acids, the hydroperoxide group of their ADAM esters was treated with (+)-Noe's reagent (13–15) in the presence of catalytic amount of p-toluene sulfonic acid in tetrahydrofuran, which resulted in the formation of peracetel derivatives of 9-anthrylmethyl, 2-hydroperoxy carboxylate. HPLC of the derivatives was done as described previously (5). Racemic 2-hydroperoxy-aliphatic acid and (R)-2-hydroperoxyaliphatic acid were synthesized as described previously (16).

**Mutagenesis—**In vitro mutagenesis was performed by using the QuikChange™ site-directed mutagenesis kit (Stratagene) with the following primers (the bases changed are underlined). His1354-Gln sense primer (5′-ACAATCGGCATCTTGCACTGGGATGATCA-3′) and antisense primer (5′-TTGGTTCATGGGTTCAGGACTGATTGATGAT-3′); His1399-Gln sense primer (5′-TTTTTATATAAGACAGACGATCCGATTTAAGCTGATT-3′) and antisense primer (5′-ATCAGAGCGTCATTTTGATCT-3′); His1927-Gln sense primer (5′-TTTCATTTAAGAACGACAGATTTTGATCTGATT-3′) and antisense primer (5′-ATCAGAGCGTCATTTTGATCT-3′); Tyr153-Tyr sense primer (5′-TTTACAGTTTTTTCAGAATGAGCTCAGTTG-3′) and antisense primer (5′-TTTACAGTTTTTTCAGAATGAGCTCAGTTG-3′); Ser383-Asp antisense primer (5′-TTTATTTATATATTGGAAGCAGAGGCGGTGAAAT-3′) and antisense primer (5′-TTGGGAGGCTCAGTCTGTTGAGGGAACAT-3′). Mutation was confirmed by DNA sequencing.

**RESULTS**

**α-Oxidation System in Rice Seedlings—**In rice seedlings, it has been postulated that selective inactivation of alcohol dehydrogenase is accountable to fatty acid α-oxidation activity (17), although it has not been known whether the activity is the same as those found in the other plants such as cucumber and bean (8, 18). When crude homogenate prepared from rice leaves was incubated with palmitic acid, n-pentadecanal was detected as a major product. The activity needed molecular oxygen (data not shown). Thus, it would be considered that rice leaves have the fatty-acid α-oxidation system. No activity could be found in dry rice seeds and seedlings grown until day 7; thereafter, it increased gradually and became highest at 30–35 days after germination (~45 nmol of n-pentadecanal formed/min/g fresh weight from palmitic acid) (Fig. 1). Most of the activity was
found in shoots, whereas roots had low activity. Sequential centrifugation revealed that the activity could be mainly recovered in a membrane fraction; however, no distinct localization in a certain organelle could be observed.

Sequence Analysis—In tobacco, the oxygenation on the α-position of fatty acids was found to be carried out by a novel oxygenase named PIOX, which has a structural resemblance with mammalian PGHS (10). We found that an EST clone (S10043, the rice EST data base in MAFF DNA Bank) derived from green shoots of rice has high sequence similarity with tobacco PIOX. The clone has an insert of 2208 bp comprising 111 bp of 5’-untranslated leader sequence and 301 bp of 3’-untranslated sequence. Because a stop codon upstream of the putative initiation codon could be found in-frame, the cDNA seemed to be full-length. Kozak consensus sequence, 5’-GCAATGGG-3’ (the initiation codon is underlined) was also found in an appropriate position. The open reading frame encodes a deduced protein of 619 amino acids with a calculated Mr of 70710 and a pI of 8.87 (Fig. 2A). The deduced amino acid sequence has a high similarity with those of tobacco and Arabidopsis PIOXs (63.6 and 61.2%, respectively). The rice sequence also shares sequence similarity with mammalian PGHSs, namely 25.7 and 31.6% amino acid identity with PGHS-1 of sheep (19) and PGHS-2 of mouse (20), respectively. Interestingly, the rice sequence also has high sequence homology with tomato Feebly gene, which is identified by transposon tagging (21). The insertional mutagenesis of the Feebly gene in tomato produced high anthocyanin levels, developed into small fragile plants, and were insensitive to the herbicide phosphinothricin. Sequence analysis with PSORT algorithm (psort.nibb.ac.jp/form.html) suggested that the rice sequence has no signal to direct the protein to a distinct organelle. As shown in Fig. 2B, in a phylogenic tree of PGHS and PIOX homologs, the rice protein locates in a family of plant PIOXs but not in the family of Feebly proteins. Both the plant proteins are apparently distinct from the mammalian PGHSs. Linoleate diol synthase of the fungus Gaecumannomyces graminis, which also has the sequence similarity with a mammalian PGHS (22), locates separately in the tree.

In sheep PGHS-1, His-309 is noted as one of the heme ligands. Tyr-385 is noted to form a tyrosyl radical, which initiates the PGHS reaction by abstracting the 13-pro-S-hydroxyl group from arachidonate. They are conserved completely within the PGHS family. They were also found to be present in the rice sequence at the appropriate positions of His-277 and Tyr-380 (Fig. 3). The amino acid sequences surrounding them are also conserved, particularly near the Tyr-380. Another histidine residue (His-383), which is also reported to be essential to PGHS activity (His-388 in sheep PGHS-1), can be found at the same array. In addition, His-207 (in sheep PGHS-1), which is also essential for the PGHS activities (23), was also conserved in the rice sequence at the position His-158. However, the amino acid residue equivalent to Ser-530 (PGHS-1), which is known as the binding site for the substrate and the target residue for many NSAIDs (24), was not found at the appropriate position in the rice sequence. Although a serine residue could be found near the one conserved in mammalian PGHSs, the amino acid sequence around the residue showed low sequence similarity; therefore, this relevance seemed to have little significance. Epidermal growth factor-like domain found in PGHSs was absent in the rice α-oxygenase sequence. In the rice sequence as well as in the other plant α-oxygenase sequences, N-glycosylation site could not be found.

Purification of α-Oxygenase—To reveal enzymatic properties of rice α-oxygenase, the corresponding cDNA was incorporated into an E. coli expression vector, and the recombinant protein was expressed and purified to apparent homogeneity on SDS-polyacrylamide gel electrophoresis (cf. Fig. 7). The recombinant protein was recovered with a membrane fraction and needed to be solubilized with a detergent. The purified α-oxygenase had a specific activity of 0.162 microkatal/mg when oxygen uptake was followed with linoleic acid as a substrate. The specific activity was slightly lower than those reported for sheep PGHSs (ranging from 0.37 to 1.3 microkatal/mg) (25, 26). The molecular mass of the recombinant protein was estimated to be 70,000 kDa on SDS-PAGE, which is in good accordance with that calculated from the sequence.

Spectral Properties of α-Oxygenase—Absorption spectrum of the purified recombinant rice α-oxygenase is shown in Fig. 4. It had a Soret absorption band with a maximum at 410 nm and shoulders at 529, 563, and 628 nm. The last one is characteristic of high spin heme species. This spectrophotometric feature was comparable with PGHS, which has the Soret α/β and charge-transfer transitions at 411, 501, and 633 nm (25, 27, 28). The molecular coefficient was 11.6 mM$^{-1}$ cm$^{-1}$ (410 nm). This value was somewhat lower than those reported for mammalian PGHSs (ranging from 123 to 165 mM$^{-1}$ cm$^{-1}$). Upon reduction of the enzyme with dithionite, only a slight change could be found at the Soret absorption band, namely the λmax blue-shifted by only 1–2 nm and the molar extinction coefficient decreased slightly. On the contrary, the α/β and charge-transfer transitions changed significantly. With the reduced form, a broad band at ~507 nm was evident in the α/β band, and clear peaks at 636 and 661 nm appeared in the charge-transfer transitions. The spectrophotometric properties of reduced form of rice α-oxygenase was totally different from those of mammalian PGHSs having peaks of 428, 530, and 558 nm for the Soret β and α bands, respectively (28). In short, the heme coordination state of ferrous form of the rice enzyme has a close resemblance with those of mammalian PGHSs, although that of ferric state is significantly different.

Product and Substrate Specificities—When the purified recombinant enzyme was reacted with linoleic acid at 25 °C, the formation of (8Z,11Z)-heptadecadienal, which is a fatty aldehyde with one methylene length shorter than the substrate, could be detected as shown in Fig. 5A. No formation of this compound could be detected when the E. coli cells harboring the expression plasmid without the insert were used instead. In algae as well as in higher plants (5), it has been reported that a long-chain fatty aldehyde would be formed through decarboxylation of the corresponding 2-hydroperoxy fatty acid (Scheme 1). 2-Hydroperoxy fatty acids are chemically unstable and have a half-life time of ~30 min in an aqueous buffer at 23 °C (11). Thus, to detect the unstable fatty acid 2-hydroperoxide, the fatty acid as a substrate was incubated with the purified enzyme on ice and the product was immediately esterified with ADAM. The resulting ADAM esters were analyzed by HPLC. When palmitic acid was incubated with the recombinant purified enzyme on ice, 2-hydroperoxy of palmitic acid was detected as a major product as shown in Fig. 5B. To elucidate the stereochemistry of the hydroperoxide group, the ADAM ester was treated with Nöe’s reagent and the derivative was separated with reversed phase HPLC analysis (5). As shown in Fig. 5C, the stereochemistry of the hydroperoxide was revealed to be (R)-configuration in almost 100% specificity. This stereochemistry is same as that of the α-oxygenation enzyme obtained with various marine algae and tobacco plant. The recombinant enzyme per se has extremely low, if any, peroxidase activity to reduce the hydroperoxide. When palmitic acid 2-hydroperoxide was incubated with the recombinant enzyme under the presence of guaiacol, which is widely used as a co-substrate for peroxidase activity of PGHSs, a little increase
FIG. 2. Deduced amino acid sequence of rice Ω-oxygenase (A) and a phylogenetic tree made with plant PIOXs, PGHSs, and Feeblys (B). Multialignment of the sequence was performed with ClustalW (hypernig.nig.ac.jp/homology/clustalw.shtml). The phylogenetic tree was made with Tree View PPC in a Phylip format. The scale bar (0.1) means 0.1 nucleotide substitutions per site. The sequences used making the tree are tobacco PIOX, rice PIOX (this study, GenBank access number AAF64042), Arabidopsis PIOX, tomato Feebly, Arabidopsis Feebly, Neurospora Feebly, mouse PGHS-2, sheep PGHS-1, coral PGHS, and Gaeumannomyces linoleate diole synthase.
in the amount of 2-hydroxide was detected. In the case of PGHSs, their peroxidase activities were intrinsically essential to complete their reaction to form prostaglandin H and also essential to make inactive ferrous form of the enzymes active through the formation of a tyrosyl radical that is needed to abstract a hydrogen atom from substrate fatty acid. Thus, the turnover rates of oxygenase pathway and peroxidase pathway are almost stoichiometrically same. In contrast, in the case of rice α-oxygenase, fatty acid 2-hydroperoxide was the major product at least under the reaction condition employed here; therefore, the rice enzyme has little need to provoke peroxidase pathway. Nonetheless, at least one catalytic turnover of the peroxidase pathway must be essential to activate the oxygenase. As the proposed mechanism of PGHS action indicated (29), a single peroxidase turnover to produce the tyrosine radical may be sufficient to initiate several hundred cyclooxygenase turnovers. We failed to detect hydroxide as one of the products formed by the rice enzyme, probably because extremely low number of peroxidase turnover was employed during its catalysis.

Within palmitic, stearic, oleic, linoleic, and α-linolenic acids, linoleic acid was revealed to be the best substrate for the rice α-oxygenase, although the other fatty acids also have substantial levels of reactivities, which indicated that (1Z,4Z)-penta-2,4-diene system is not the essential prerequisite to be a substrate. With linoleic acid as a substrate, there is no change in the absorbance between 210 and 350 nm including absorption at 234 nm corresponding to the formation of a conjugated diene hydroperoxy moiety during the reaction. This finding indicated that rice α-oxygenase could not abstract the bisallylic proton to form conjugated diene hydroperoxide but exclusively abstract α-proton.

The Reaction Progress Curves of α-Oxygenase—When the reaction of recombinant rice α-oxygenase was continuously followed by using an oxygen electrode, the kinetic lag phase was observed as shown in Fig. 6. This finding indicated that the native form of recombinant rice α-oxygenase is active and needs catalytic activation during its reaction to ensure its maximum activity. This was consistent with the proposed catalytic mechanism of PGHSs in which the ferrous form is a native inactive form (30). The ferrous form is oxidized by hydroperoxides to form species analogous to compound I of horseradish peroxidase, which in turn accelerates the formation of a tyrosyl radical that is essential to the abstraction of the hydrogen atom of the substrate arachidonic acid (31). If this is also the case...
with the rice enzyme, the addition of hydroperoxide must abolish the lag phase of the reaction. As shown with Fig. 6, trace B, the addition of tert-butyl hydroperoxide apparently shortened the lag of the reaction of \( \alpha \)-oxygenase; however, the maximal rate of oxygen consumption was unaffected by the addition.

The enantioselective \( \alpha \)-hydroperoxylation of long-chain fatty acids in plants. Long-chain fatty acids undergo \( \alpha \)-oxidation to give 2-hydroperoxy fatty acids as intermediates, which then degrade to aldehydes non-enzymatically with releasing \( \text{CO}_2 \).

FIG. 5. HPLC detection of the products formed by purified \( \alpha \)-oxygenase. A, the purified enzyme was incubated with linoleic acid as a substrate for 10 min at 25 °C. The product, \((8Z,11Z)\)-heptadecadienal, was converted to the corresponding 2,4-dinitrophenylhydrazone derivative and analyzed by HPLC. B, after the reaction of recombinant \( \alpha \)-oxygenase with palmitic acid for 15 min on ice, the product formed is analyzed as fluorogenic ADAM derivative. C, 2-hydroperoxyhexadecanoic acid formed by the enzyme action was ADAM-esterified and converted into its peracetal derivative and then separated with reversed-phase HPLC (trace a). Each enantiomer can be separated when racemic 2-hydroperoxide was analyzed (trace b). Under the condition employed here, \((R)\)-enantiomer elutes fast and then \((S)\)-enantiomer follows (5). Co-injection of the racemic hydroperoxide and enzymatic product enlarged solely the peak corresponding to \((R)\)-enantiomer (trace c).

FIG. 6. Effect of hydroperoxide on the kinetics of \( \alpha \)-oxygenase activity. The purified recombinant enzyme was preincubated with or without 20 mM tert-butyl hydroperoxide at 25 °C for 2 min, and then the reaction was started by the addition of the substrate, linoleic acid. Trace A, without hydroperoxide; trace B, with the hydroperoxide.

Reaction Mechanism of Rice \( \alpha \)-Oxygenase

with the rice enzyme, the addition of hydroperoxide must abolish the lag phase of the reaction. As shown with Fig. 6, trace B, the addition of tert-butyl hydroperoxide apparently shortened the lag of the reaction of \( \alpha \)-oxygenase; however, the maximal rate of oxygen consumption was unaffected by the addition.

Inhibition of \( \alpha \)-Oxidation Activity—As noted with plant \( \alpha \)-oxidation system (8), imidazole was a potent reversible inhibitor for the rice \( \alpha \)-oxygenase, and the \( K_i \) value was determined to be 0.046 mM (Table I). It is well known that imidazole can bind to Fe in most heme proteins (32) that results in the inactivation of the function of the heme protein. On the contrary, no inhibition of ram seminal PGHS has been reported even at high imidazole levels as much as \( \geq 500 \text{ mM} \) (27), although the addition of imidazole changed the absorption spectrum of PGHS, which indicated that imidazole could bind to the PGHS. This addition was again a big difference in the properties of rice \( \alpha \)-oxygenase and mammalian PGHSs. Nonsteroidal anti-inflammatory drugs such as aspirin and flurbiprofen are known to be irreversible inactivators of PGHSs \( \text{in vitro} \). Ser-530 in sheep PGHS is known to be the target of aspirin to exert its inhibitory activity through acetylation of the hydroxy group (30). This residue is thought to have an important role in binding the substrate of PGHS, arachidonic acid. For example, the estimated \( K_i \) value of aspirin was reported to be 14 mM with sheep PGHS, and the preincubation of the enzyme with 1 \( \mu \text{M} \) flurbiprofen for 1 min decreased the activity to almost 30% of the initial activity (33). As shown in Table I, aspirin and flurbiprofen were not potent inhibitors for rice \( \alpha \)-oxygenase activity.
Inhibition of α-oxygenase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Activity</th>
<th>K_i value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.161 ± 0.017</td>
<td>—</td>
</tr>
<tr>
<td>Aspirin</td>
<td>30</td>
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<td>na</td>
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<tr>
<td></td>
<td>50</td>
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**Discussion**

This study showed that rice α-oxygenase is a heme enzyme having a structural similarity with mammalian PGHSs. The heme moiety seems to be essential to the activity, because exchange of the histidine residues possibly involved in heme binding abolished the activity. The native form of the enzyme is inactive and needs to be activated by hydroperoxide probably by its own product, fatty acid 2-hydroperoxide. The activation may result in the formation of a tyrosyl radical, which in turn abstracts a hydrogen atom from the methylene unit in the substrate to support stereochemical oxygenation of the carbon. As a result, hydroperoxide group is introduced on the C2-configuration in a stereochemically unequivocal manner. This catalytic course of oxygenation of fatty acids is similar to that of PGHSs; thus, in this context, the catalytic mechanism proposed and widely accepted for mammalian PGHSs can be applied to the rice oxygenase. However, there still is an outstanding difference between rice oxygenase and PGHSs. PGHS is a bifunctional enzyme and can carry out both the cyclooxygenase and peroxidase activities; on the contrary, little peroxidase activity can be found with rice α-oxygenase. In the case of PGHS, tight coordination of both the activities is essential to complete its reaction to form PGH. However, both of the activities function independently because Mn^3+-protoporphyrin-reconstituted PGHS, for example, lacks appreciable peroxidase activity but still exhibits functional cyclooxygenase activity. Apparently, the structure around the heme moiety, especially at the peroxidase site proposed with PGHS (31) must be different between rice α-oxygenase and PGHS. The fact that spectrophotometric features of the reduced form of α-oxygenase and PGHS totally different from each other might be a result of this difference in the active site.

Unexpectedly, NSAIDs showed little effect on the rice α-oxygenase activity. This low effect may be caused because 1) NSAIDs could not reach the serine residue in the active site or 2) there is no serine residue in the active site. Although not complete, the conserved serine residue can be found in the plant α-oxygenases in a position near the active site serine in mammalian PGHSs. Furthermore, site-directed mutagenesis
of Ser-558 in rice α-oxygenase resulted in an inactive enzyme. Thus, the former possibility seems to be more sensible from which we can assume that the structure of the substrate recognition site is also diverged from that of PGHS. This finding is rational, because rice α-oxygenase oxygenize only the C₂ position of the fatty acid and a pentadiene system occurring in polyunsaturated fatty acid is not the prerequisite for the activity. To obtain concrete evidence for the assumption depicted with this study, further extensive study is needed.

REFERENCES