Purification and Catalytic Activities of the Two Domains of the Allene Oxide Synthase-Lipoxygenase Fusion Protein of the Coral *Plexaura homomalla*

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The conversion of fatty acid hydroperoxides to allene epoxides is catalyzed by a cytochrome P450 in plants and, in coral, by a 43-kDa catalase-related hemoprotein fused to the lipoxygenase that synthesizes the 8R-hydroperoxyeicosatetraenoic acid (8R-HPETE) substrate. We have expressed the separate lipoxygenase and allene oxide synthase (AOS) domains of the coral protein in *Escherichia coli* (BL21 cells) and purified the proteins; this system gives high expression (1.5 and 0.3 μmol/liter, respectively) of catalytically active enzymes. Both domains show fast reaction kinetics. Catalytic activity of the lipoxygenase domain is stimulated 5-fold by high concentrations of monovalent cations (500 mM Na⁺, Li⁺, or K⁺), and an additional 5-fold by 10 mM Ca²⁺. The resulting rates of reaction are ~300 turnovers/s, 1–2 orders of magnitude faster than mammalian lipoxygenases. This makes the coral lipoxygenase well suited for partnership with the AOS domain, which shows maximum rates of ~1400 turnovers/s in the conversion of 8R-HPETE to the allene oxide. Some unusual catalytic activities of the two domains are described. The lipoxygenase domain converts 20.3-cis partly to the bis-allylic hydroperoxide (10-hydroperoxyeicosa-8,11,14-trienoic acid). Metabolism of the preferred substrate of the AOS domain, 8R-HPETE, is inhibited by the enantiomer 8S-HPETE. Although the AOS domain has homology to catalase in primary structure, it is completely lacking in catalatic action on H₂O₂; catalase itself, as expected from its preference for small hydroperoxides, is ineffective in allene oxide synthesis from 8R-HPETE.

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The Caribbean coral *Plexaura homomalla* is famous for its high content of prostaglandin esters, which constitute 2-3% of the coral dry weight (1). In the early 1970s, at a time when practical chemical syntheses of the prostaglandins were still under development, *P. homomalla* was used as a commercial source of these mammalian hormones. This practical interest led in turn to investigation of the coral prostaglandin synthase. The issue of the biochemical pathway turned out to be difficult to tackle in *P. homomalla* itself, because, for reasons that remain unclear, prostaglandin synthesis is not observed in *in vitro* preparations of this particular coral (2). Using another species, the Arctic coral *Gersemia fruticosa*, a body of evidence indicates an endoperoxide pathway akin to the mammalian cyclooxygenase route (3, 4).

Although it has proved difficult to detect prostaglandin synthesis using *in vitro* preparations of *P. homomalla*, the metabolism of added arachidonic acid is readily observed (2, 5). There is very high 8R-lipoxygenase activity, sufficient to consume perhaps 1 mM arachidonic acid in a 1:10 tissue/buffer homogenate. At least two distinct lipoxygenases account for this activity. One is a predominantly soluble enzyme, essentially a typical lipoxygenase in terms of catalytic properties, primary structure, and molecular weight (6). The second enzyme, the subject of the present investigation, is unique among reported lipoxygenases in that it occurs as a natural fusion protein with an extra 43-kDa N-terminal domain that functions as an allene oxide synthase (7).

Allene oxides are unstable epoxides formed from lipoxygenase-derived fatty acid hydroperoxides. Biosynthesis has been detected in both plants and marine invertebrates (8, 9). Allene oxides can cyclize to form five-membered carbon rings, and initially this was suspected to be part of the coral route to the prostaglandins (2, 5). In plants, the enzymatic cyclization of an allene oxide derived from 13-hydroperoxylinolenic acid is established as a step in biosynthesis of the cyclopentanone hormone, jasmonic acid (10). Although the allene oxide pathway has not been substantiated for prostaglandin synthesis in coral, it may constitute the route to other prostanooid-related products such as the clavulones and punaglandins of other corals (5, 11, 12). There is also the implication of other as yet undefined roles for allene oxides. In starfish oocytes, for example, allene oxide synthesis occurs in the absence of enzymatic cyclization of the epoxy (13), and this also appears to be the case in *P. homomalla* (2).

The enzyme involved in allene oxide synthesis in plants is a member of the cytochrome P450 family of hemoproteins and is designated as CYP74A (14). In contrast to the typical P450 monoxygenase, this allene oxide synthase (AOS)1 does not require molecular oxygen or reducing equivalents from NADPH. The ferric form of the hemoprotein converts the 13S-hydroperoxides of linoleic and linolenic acids to the corresponding allene oxides with a turnover number of ~1000/s (15). The plant AOS is closely related to the aldehyde-forming hydroperoxide lyase, another plant cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides (16).

The plant and coral AOS enzymes catalyze reactions with identical chemistry. It was unexpected, therefore, when cloning

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1 The abbreviations used are: AOS, allene oxide synthase; H(P)ETE, hydro(per)oxyeicosatetraenoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl; LOX, lipoxygenase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; AU, absorbance unit; NTA, nitrilotriacetic acid.

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of the second lipoxygenase from *P. homomalla* led to character-
zation of a novel type of alene oxide synthase unrelated to the
cytochrome P450 s (7). The cDNA of the lipoxygenase-
related transcript encoded a protein with a predicted molecular
mass of 122 kDa. Sequencing revealed a C-terminal lipoxy-
genase domain of 79 kDa and a unique N-terminal domain of 43
kDAs with some weak homology to catalase. Expression of the
fusion protein and of the two separate domains established the
8R-lipoxygenase activity of the C-terminal 79 kDa and the AOS
activity of the catalase-related N-terminal domain (7). The
lipoxygenase forms a specific 8R-hydroperoxo fatty acid, the
substrate of the AOS domain. A more detailed study of the two
domains of this unique fusion protein is described here.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid was purchased from NuChek Prep Inc
(Elysian, MN), and [1,14C]arachidonic acid from NEN Life Science
Products. HPETE and HETE standards were prepared by vitamin
E-controlled auto-oxidation (17), and 9R-HPETE was synthesized using
acetone powder extracts of *P. homomalla* (2).

**Preparation of Constructs for Bacterial Expression**—To prepare the
AOS domain with a C-terminal His tag, the AOS domain was cloned
into pET3a with the 5′-GGGGAAGATCCGGTCACTGATGTAAC-3′
(GCC
ATG
ACT
GGT
GGA
CAG
CAA
ATG
GGT
GCC
GGC
ACC
ATG-3′,
with the last codon being the start of the wild type enzyme. To ligate the
3′ end of the AOS domain into pET3a, the vector was cut at the unique
Clal site. The 3′ end of the AOS sequence was modified by the addition
of four histidines encoded after amino acid 373, followed by a step codon
and Clal restriction site, -AAT CAT CAC CAT CAC TAA ATC GAT-3′,
with the two last codons being the restriction site for Clal; the step
codon was encoded as TAA and not TAG, because the enzyme Clal is
dam-sensitive, and it cannot cut when the restriction site is preceded by
a G (as in TAG), in which case the first adenine of the restriction site
would be methylated. The 8R-lipoxygenase domain was cloned into
pET3a at the BamHI site and expressed with 14 amino acids from the
vector (as above) followed by a His4 tag (…ATG CAT CAC CAT CAC
AAT GCT-…), with the last two codons representing the
start of the wild type lipoxygenase sequence.

**Bacterial Expression of the Separate Lipoxygenase and AOS Do-
 mains**—The two domains were expressed separately in BL21 cells using
a modified expression methodology described by Hoffman et al. (18).
A typical preparation of a 50-ml culture was carried out as follows; 1 ml
of LB medium containing 400 μg/ml ampicillin was inoculated with a
single colony of AOS-His in BL21 cells and grown at 37 °C at 250 rpm
for 4 h. An aliquot (200 μl) of this culture was used to inoculate 10 ml
of fresh LB medium (with 400 μg/ml ampicillin) and re-grown for 3 h;
the culture was then diluted with 40 ml of TB containing 100 μg/ml
ampicillin and grown at 28 °C, 250 rpm for 24 h. The cells were spun
down at 5000 rpm (≈6000 × g) for 10 min in a Sorvall RC-3 centrifuge,
washed with 40 ml of 50 mM Tris-HCl buffer, pH 7.5, adjusted to 500
rpm for 10 min, and resuspended in 10 ml of TSE buffer (100 mM Tris
acetate, pH 7.6, 500 mM sucrose, 0.5 mM EDTA) containing 1 mg/ml
lysozyme and kept on ice for 30 min. The spheroplasts were then spun
down at 5000 rpm for 15 min, resuspended in 10 ml of spheroplast
buffer, and frozen at −80 °C. When aliquots were thawed (on ice),
phenylmethylsulfonyl fluoride was added at a final concentration of
1 mM. The spheroplasts were then sonicated twice for 30 s using a model
50 Sonic Dismembrator (Fisher Scientific) at a setting of 2. The result-
ing membranes were spun down at 100,000 × g for 90 min at 4 °C. AOS
activity is present in the supernatant. The same procedure was used for
the lipoxygenase domain, except that it was recovered in the 100,000 ×
supernatant after solubilization using 0.2% Emulphogene BC-720™
(polyoxyethylene 10 tridecyl ether, Sigma).

**Purification of His-tagged Proteins**—The histidine-tagged aleene
oxide synthase (His-AOS) was purified following the protocol of Imai et al.
(19). The 150,000 × g supernatant was loaded on a nickel-NTA column
(0.5 ml bed volume, Qiagen) equilibrated with 50 mM potassium phos-
phate buffer, pH 7.2, 500 mM NaCl at 0.5 ml/min. The column was then
washed with the equilibration buffer and the nonspecific binding pro-
teins were eluted using 50 mM potassium phosphate buffer, pH 7.2, 500
mM NaCl, 50 mM glycine. The His-AOS was then eluted with 50 mM
potassium phosphate buffer, pH 7.2, 50 mM NaCl, 40 mM 1-histidine.
Fractions of 0.5 ml were collected and assayed for the AOS activity.
The positive fractions were dialyzed overnight against 50 mM Tri-His-HCl
buffer, pH 7.9, using a microdialyzer (Pierce). For the lipoxygenase
purification, 0.2% Emulphogene BC-720™ was included throughout
the procedure. The purity of the enzyme preparations was determined
by SDS-PAGE and Coomassie Blue staining.

**Quantitation of the Purified Proteins**—Colorimetric protein assay
using BCA reagent (Pierce) was carried out according to the manufac-
turer’s instructions using bovine serum albumin as a standard. For the
lipoxygenase, colorimetric assay of a 100 μM substrate and typically in
50 mM Tris, pH 8.0, with additions of salts as described under “Results.”
Unlabeled substrates were used for spectrophotometric assays (monitoring increase in UV absorbance at 255 nm); for structural analysis of products from C20 fatty acids, 14C-labeled 20:3ω6, 20:4ω6, or 20:5ω3 were included in the
incubations. Products were extracted either using the Bligh and Dyer
procedure (22) or using methylene chloride. For complete recovery of
the more polar products the aqueous phase was acidified to <pH 5 prior
to extraction. To optimize recovery of acid-labile products from 20:3ω6,
the pH of the solutions for extraction were kept higher than 5 and the
organic extract was washed with water (or Bligh and Dyer upper phase)
prior to evaporation. Extracts were kept in methanol at −20 °C under
argon prior to analysis.

**Spectrophotometric Enzyme Assays**—The AOS domain was assayed
by following the decrease in absorbance at 235 nm of the substrate
hydroperoxides using a Beckman DU-7 spectrophotometer essentially
as described (21). Lipoxygenase activity was assayed using the increase in
absorbance at 235 nm.

**HPLC Analysis of Products**—Extracts were analyzed initially by
RP-HPLC using a Beckman Ultrasphere ODS 55 or Waters Symmetry
C18 columns (25 × 0.46 cm) with solvents of methanol/water/acetic
acid, typically in the proportions 90/10/0.1 (v/v/v) for a quick screen of
the products formed, and 80/20/0.1 (v/v/v) for more detailed analysis
and collection of individual compounds. Triphenylphosphine in metha-
oletherountved Suite

**GC-MS Analyses**—Methyl esters were prepared with diazomethane
and trimethylsilyl (TMS) derivatives using N,O-bis(trimethylsilyl)tri-
fluoroacetamide/pyridine. Products were hydrogenated prior to silylation
in 100 μl of ethanol using palladium of carbon (1–2 mg) and bubbling with
hydrogen for 2–5 min; water was then added and the products extracted
with ethyl acetate. GC-MS analyses were carried out in the electron
impact mode (70 eV) using a Finnigan Incos 50 mass spectrometer
coupled to a Hewlett-Packard 5890 gas chromatograph equipped
with a SPB-1 fused silica capillary column (5 or 15 m × 0.25 mm
internal diameter). Samples were injected at 190 °C and the tempera-
ture was subsequently programmed to 300 °C at 10 °C/min.

**Stopped-flow Data Acquisition and Analysis**—The initial velocity
of the AOS was determined on an Applied Photophysics Ltd. Model
SX17TM stopped-flow spectrometer operated in the absorption mode at
25 °C. The wavelength was set at 235 nm with a monochromator
grating of 56 μm Tri-HCl, pH 7.9. The AOS was used at a final concentra-
tion of 4.4 × 10−6 M with final substrate concentrations ranging from 0 to 70
μM. Measurements at higher substrate concentrations (120–160 μM)
were made using a 0.2-μm path length flow cell with the concentra-
tion of AOS decreased to 1.1 × 10−4 M; the values obtained were
normalized to a 1-cm path length. Following each injection, the decrease in absorb
The solubilized lipoxygenase (with N-terminal His tag) was purified as described under “Experimental Procedures.” The purity of the enzyme in the elution fractions 3–6 was determined by 10% SDS-PAGE and Coomassie Blue staining. The arrow indicates the lipoxygenase. MW represents the molecular mass markers in kDa.

The LOX domain showed maximum rates of reaction at pH 8.0. Such an effect might be due to the difference in the ionic strength of the two buffers (25). Indeed, addition of inorganic cations normalized the difference between the two buffers and gave an additional increase in catalytic activity (Fig. 3A). By contrast, a higher concentration of Tris-HCl (0.5 M) was ineffective. The effect of inorganic cations reached a maximum at a concentration of 500 mM; the enzyme did not discriminate markedly between KCl, LiCl, or NaCl (Fig. 3A). Although the enzyme was not calcium-dependent (2 mM EDTA had no effect on catalysis), activity was further enhanced by addition of millimolar concentrations of CaCl₂ (Fig. 3B). By contrast, MgCl₂ (10 mM) had no effect. Although the enzymatic activity is increased in the presence of 500 mM NaCl, the substrate inhibition displayed by the lipoxygenase at arachidonic acid concentrations over 50 μM was not markedly changed (Fig. 4). The enzyme exhibited a turnover number of 300–350/s in pH 8 Tris containing 500 mM NaCl and 10 mM CaCl₂.

**RESULTS**

**Expression of the Lipoxygenase Domain**—The primary structure of the lipoxygenase domain of the coral fusion protein shows an alignment with mammalian lipoxygenases such that position 372 of the fusion protein aligns with the initiating methionine of mammalian lipoxygenases. A construct of the coral lipoxygenase sequence (amino acids 372–1067) was prepared in pET3a vector and including a His tag sequence at the 5’ end. Expression of the lipoxygenase domain in *E. coli* (BL21 cells) gave highly active enzyme, with the activity recovered in the 10,000 × g pellet of sonicated cells. The lipoxygenase was solubilized using Emulphogene BC-720™ detergent (0.1%); it could then be purified by affinity chromatography on a nickel-NTA column (Fig. 1). The enzyme was fairly stable in the column eluant (50 mM Tris-HCl, pH 7.9, 0.2% Emulphogene); it retained about half its original activity after 3 months at 4 °C. The LOX domain showed maximum rates of reaction at pH 7–7.5 (Fig. 2). Spectrophotometric reactions were often performed at pH 8 to improve the solubility of substrate and hence the clarity of the solution.

**Salt Dependence**—The lipoxygenase domain exhibited some interesting and unusual kinetic properties. Initially, we noted substantially higher catalytic activity in 100 mM sodium phosphate buffer compared with 100 mM Tris-HCl (both buffers at pH 8.0). Such an effect might be due to the difference in the preference for C20 or C22 highly polyunsaturated fatty acids. The C18 fatty acids, linoleic acid, were not substrates. The best substrate was 22.6 μM 10,12-HPETE. The enzyme exhibited a turnover number of 300–350/s in pH 8 Tris containing 500 mM NaCl and 10 mM CaCl₂.

**Inactivation of the Lipoxygenase**—In the absence of substrate, the lipoxygenase domain rapidly lost activity upon dilution from the concentrated stock solution kept in nickel column elution buffer. The key ingredient for enzyme stability in the nickel column elution buffer was the 0.5% concentration of Emulphogene detergent. The lipoxygenase, however, was not catalytically active in the presence of 0.1–0.5% Emulphogene. Accordingly, this detergent could not be used to prolong the lifetime of the enzyme under conditions suitable for metabolism of substrate. The enzyme also was inhibited in its catalytic activity by other detergents including octyl glucoside (25 mM), CHAPS (10 mM), reduced Triton X-100 (0.1%), Nonidet P-40 (0.29 mM), Triton X-100 (0.24 mM), and sodium cholate (14 mM). Glycerol at 10–20% (v/v) slightly increased the rate of reaction with arachidonic acid without substantially prolonging the lifetime of the active enzyme. Concentrations of 40% glycerol slowed the reaction rate but also preserved the catalytically active enzyme and, remarkably, led to the highest conversion of substrate.

**Substrate Specificity**—The lipoxygenase domain showed a preference for C20 or C22 highly polyunsaturated fatty acids. The C18 fatty acids, linoleic acid, α-linolenic acid, and γ-linolenic acid, were not substrates. The best substrate was 22.6 μM (designated as 100% relative reaction rate), followed by 20.5 μM (85%), 20.4 μM (70%), and 20.3 μM (35%). The major hydroperoxy
hexaenoic acid. The parent product is the 10-hydroperoxydocosa-4,7,11,13,16,19-hexaenoic acid. Allene Oxide Synthase: pH Dependence, Rates of Reaction, and Inactivation (Figs. 6 and 7)—The coral AOS was active over the range pH 5–9, and it exhibited a broad pH optimum at pH 7.5. The initial rate of reaction of the coral AOS domain decreased with increasing pH, was unchanged from pH 5 to 7.9, and with ( ) 0.5 M NaCl. After checking that the absorbance at 235 nm was constant, the lipoxygenase (18.6 pmol) was added and the increase in absorbance was monitored. The rate of reaction was represented as amount of 8R-HPETE formed/min/pmol of enzyme.

FIG. 3. Effects of salts on activity of the lipoxygenase domain. Panel A, reaction rates with arachidonic acid (30 μM) were measured as described in Fig. 2 legend in 500 μl of 50 mM Tris-HCl, pH 7.9, containing different concentrations of NaCl ( ), KCl ( ), or LiCl ( ). Panel B, effects of CaCl2 (tested in the presence of 500 mM NaCl). In the experiments with millimolar concentrations of calcium ions, Emulphogene detergent (0.01% final concentration) was used to prevent precipitation of the calcium salts of the fatty acid substrate and thus permit use of the 235-nm UV absorbance assay.

FIG. 4. Lipoxigenase activity as a function of arachidonic acid concentration. Arachidonic acid at concentrations ranging from 6 to 120 μM was incubated in a cuvette with 500 μl of 50 mM Tris-HCl, pH 7.9, and with ( ) or without ( ) 0.5 mM NaCl. After checking that the absorbance at 235 nm was constant, the lipoxigenase (18.6 pmol) was added and the increase in absorbance was monitored. The rate of reaction was represented as amount of 8R-HPETE formed/min/pmol of enzyme.

FIG. 5. Metabolism of arachidonic and dihomo-γ-linolenic acids by the coral lipoxygenase domain. Reversed-phase HPLC analyses of the triphenylphosphine-reduced extracts from reaction of arachidonic acid (panel A) and dihomo-γ-linolenic acid (panel B) with the coral lipoxygenase domain. The samples were analyzed using a Waters C18 symmetry column (25 × 0.46 cm) and the solvent of methanol/water/glacial acetic acid in the proportions 80:20:0.01 v/v/v using a flow rate of 1.1 ml/min in panel A and 0.9 ml/min in panel B. Radioactivity was monitored on-line using a Packard Radiomatic Flo-One beta detector. 8-OH-20.3α6, 8-hydroxyeicos-9E,11Z,14Z-trienoic acid.

products of these fatty acids were, in turn, substrates of the AOS domain.

Product Analysis—Reactions with 100 μM substrate were conducted at room temperature in a UV cuvette while monitoring at 235 nm. After completion of reaction (1–2 min) the products were extracted, reduced with triphenylphosphine, and analyzed by HPLC, UV spectroscopy, and GC-MS. Arachidonic acid was converted to 8R-HPETE (Fig. 5A). Similarly, 20.5αδ and 22.6δω were converted to single products, identified by GC-MS analysis of the triphenylphosphine-reduced, hydrogenated methyl ester TMS ether derivatives. The hydrogenated product from 20.5αδ gave an identical mass spectrum to hydrogenated 8R-HPETE (26), and thus the enzymatic product is 8-hydroperoxyeicos-5,9,11,14,17-pentaeenoic acid. The hydrogenated product from 22.6δω showed prominent α-cleavage ions at m/z 271 (C10-C10) and m/z 273 (C10-C12), and thus the parent product is the 10-hydroperoxydocosa-4,7,11,13,16,19-hexaenoic acid.

Reaction with 20.3αδ6 gave a major 8-hydroperoxy product, as expected, and three minor products designated as A (2–5% of products in different experiments), B (2–5%), and C (5%) (Fig. 5B). Product A was not identified. Product B showed only end absorbance in the UV and a shorter retention time on RP-HPLC than 15-hydroxyeicosatrienoic acid, which is the earliest eluting of the four conjugated diene-containing monohydroxy derivatives of 20.3αδ6. On normal phase-HPLC, product B chromatographed just after 11-hydroxyeicosatrienoic acid. Product B was hydrogenated and analyzed by GC-MS in the electron impact mode as the TMS ester TMS ether derivative. The mass spectrum showed a base peak at m/z 73 and prominent high mass ions at m/z 457 (M – 15, 2% relative abundance to the base peak at m/z 73) and m/z 441 (M – 31, 2%). Besides the ions below 100 atomic mass units, the two most prominent ions were m/z 331 (C18-C8, 27%) and m/z 243 (C8-C20, 24%), consistent with α-cleavage at a C10 hydroxyl (27). The mass spectrum indicates a 10-hydroxyeicosanoic acid structure of the hydrogenated product B. In accord with this result, the UV characteristics and chromatographic behavior of the parent non-hydrogenated product B correspond to the known properties of the arachidonic acid product, 10-HETE (27). Thus, product B is identified as 10-hydroxyeicos-8,11,14-trienoic acid.

The third minor derivative, product C, had a conjugated diene ωmax at 235 nm; cf. 236 nm for the major 8-hydroxy 20.3αδ6 and a HPLC retention time consistent with 11-hydroxyeicosatrienoic acid. After methylation and hydrogenation, it was positively identified by GC-MS as the 11-hydroxy derivative (26), and thus the parent compound C is 11-hydroxyeicos-8,12,14-trienoic acid.

Expression of the AOS Domain—Expression of the full-length fusion protein or of the lipoxygenase domain in E. coli gave insoluble proteins that were recovered in the 10,000 or 150,000 g pellets of bacterial sonicates. In contrast, expression of the allene oxide synthase domain (amino acids 1–373 inclusive) gave a soluble protein. A C-terminal histidine tag sequence inserted in the open reading frame allowed a straightforward purification of the allene oxide synthase using affinity chromatography on a nickel-NTA column (7). Expression level was high (~1.5 μmol/liter), sufficient that the bacterial pellet exhibited a distinct greenish tinge.

Allene Oxide Synthase: pH Dependence, Rates of Reaction, and Inactivation (Figs. 6 and 7)—The coral AOS was active over the range pH 5–9, and it exhibited a broad pH optimum at around pH 7–8 (Fig. 7). At pH 7.5, the initial rate of reaction of the coral allene oxide synthase was estimated as ~1400 turnovers/s (84,550 ± 5090 min⁻¹) with a Km of 45.3 ± 7.5 μM as determined by disappearance of the conjugated diene chro-
Substrate Specificity of Coral AOS—Comparison of the reaction of 8R-HPETE and 8RS-HPETE gave the surprising finding that the presence of 8S-HPETE interfered with the reaction of the natural 8R-HPETE substrate. The racemic 8-hydroperoxide showed an initial rate of reaction of less than 10% of the initial rate with 8R-hydroperoxide. Both substrates showed rapid enzyme inactivation; under the conditions tested, the 8R stopped reacting after an absorbance decrease of 0.75 AU, while reaction with the racemic material ceased after an absorbance decrease of only 0.1 AU. Co-incubation with a comparable concentration of the hydroxy derivative, 8RS-HETE (30 μM), did not affect the rate of reaction with 8R-HPETE.

Investigation of the selectivity for different regio-isomers indicated that other HPETEs showed weak but measurable rates of reaction with the coral AOS domain; 8RS- and 11RS-HPETEs reacted similarly (each equivalent to about 8% of the rate with 8R-HPETE). Other racemic HPETEs reacted at 2% or less of the rate with 8R-HPETE, in the order 5RS > 12RS > 8RS-HETE.

Comparison of Catalase and AOS Activities of the Respective Enzymes—Using the decrease in absorbance at 240 nm to follow disappearance of H₂O₂, the coral AOS showed no reaction (0.00 AU change/min) at enzyme concentrations 100-fold higher than were used to produce rates of disappearance of 8R-HPETE of ~2 AU/min.

At concentrations of bovine catalase used to observe its reaction with H₂O₂, the enzyme produced no decrease in the UV absorbance of 8R-HPETE. At 1000-fold higher concentrations, catalase induced a slight decrease in UV absorbance of 8R-HPETE (0.07 AU/min). This very weak effect is likely attributed to nonspecific heme-catalyzed degradation through formation of alkoxyl radicals, as reported previously using 12-LPETO substrate (28).

**DISCUSSION**

An objective in expressing the two separate domains of the P. homomalla fusion protein was to allow study of their individual properties and catalytic activities. The isolated coral lipoxygenase domain is insoluble, similar to the intact fusion protein, but it could be recovered in good yields after solubilization with nonionic detergent. The isolated AOS domain is found to be soluble, like catalase itself, and in contrast to the membrane-associated CYP74 P450 enzymes with AOS activity. Both do-

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**FIG. 6. Initial velocity versus substrate concentration plot for the allene oxide synthase.** The initial velocity of the AOS was determined on a stopped-flow spectrometer as indicated under “Experimental Procedures.” The value at each substrate concentration was the average of seven individual experiments. The data were fit to a linear equation to determine the initial velocity of the enzyme for each substrate concentration. The velocities obtained were then plotted against the substrate concentration, and the $K_m$ and $V_{max}$ were calculated by using the double-reciprocal Lineweaver-Burk transformation.

**FIG. 7. pH activity profile of the coral AOS domain.** Arachidonic acid 30 μM was incubated in a cuvette with 500 μl of buffer (▪, Tris-HCl; ▲, potassium phosphate; ×, citrate phosphate; *, glycine sodium hydroxide) at the indicated pH. After the absorbance at 235 nm was constant, the allene oxide synthase was added and the decreased of absorbance was monitored. The rate of reaction was represented as the difference of absorbance (OD) at 235 nm normalized to the maximum observed at pH 7.0.

**FIG. 8. Rapid inactivation of the coral AOS: comparison with plant AOS (CYP74A).** Rates of reaction were monitored by recording the disappearance of substrate as the decrease in absorbance at 235 nm. Trace A, flaxseed AOS (acetone powder preparation) reacting with its preferred substrate 13S-hydropperoxylinolenic acid (50 μM); trace B, the coral AOS domain reacting with 50 μM 8R-HPETE; trace C, same as B, followed by a second addition of coral AOS enzyme. AUFS, absorbance units at full scale.
mains expressed well in E. coli, were readily purified with the aid of the His tag sequences, and retained activity for months at 4 °C.

The catalytic activity of the lipoxygenase domain is strongly influenced by the presence of inorganic cations. Monovalent cations, Li⁺, Na⁺, or K⁺ have a major activating effect, and, in addition, millimolar concentrations of calcium ions strongly stimulate the rate of reaction. In the absence of added calcium, EDTA has no effect on the basal catalytic activity. Thus, micromolar levels of calcium are ineffective and it appears that the enzyme is not calcium-requiring per se; the salts may affect the conformation of the enzyme, and they might also influence the solubility or aggregation of substrate. Conceivably this might help recreate a more natural presentation of substrate for the coral lipoxygenase domain.

In its natural marine environment, P. homomalla is osmotically equilibrated with sea water, which contains, among other ions, 0.5 M NaCl and 5–10 mM CaCl₂ (29, 30). Presumably the interior environment of the cell will contain ~0.5 M K⁺ in place of Na⁺; either is effective in stimulating the 8R-lipoxygenase domain. The free cytosolic calcium ion concentration will be nanomolar, as in typical plant and animal cells, while the total intracellular concentration of calcium may be 5–10 mM on average (29, 31) and perhaps 10-fold higher in certain subcellular compartments (31). Whether this bound calcium is “available” for modifying the enzyme activity is unclear. As an insoluble protein, the lipoxygenase domain could be membrane associated and it may normally acquire substrate while in contact with a calcium-rich compartment.

An effect of NaCl on the metabolism of arachidonic acid by extracts of P. homomalla was reported originally in connection with studies on the biosynthesis of prostaglandins (32). In retrospect it may have been lipoxygenase activity that was detected in this early work. Subsequently, a stimulatory effect of 1 M NaCl was observed on the conversion of 15-HETE to 8,15-di-H(P)ETE in P. homomalla (33). Although the metabolism of 15-HETE was not examined in the present study, the reported effect on 15-HETE metabolism may involve the same P. homomalla 8R-lipoxygenase. Hamberg and Gerwick (25) found a sodium-dependent 12-lipoxygenase activity in the marine alga Gracilaria foliifera. This enzyme showed a distinctly different profile of ion selectivity from the P. homomalla 8R-lipoxygenase domain; with the algal enzyme 0.8 mM Na⁺ gave a 20-fold stimulation, Li⁺ was half as effective, Mg²⁺ was 10% effective, and K⁺, Ca²⁺, and several other inorganic cations had no effect (25). Under optimal conditions for stimulation of the P. homomalla lipoxygenase domain (0.5 mM NaCl, 10 mM CaCl₂), the turnover numbers of the enzyme match those of the fastest known lipoxygenase, the L-1 isoyme of the soybean lipoxygenase. The values for both enzymes are around 300–350 turnovers per second per enzyme molecule (20,000 min⁻¹) (34). The reticulocyte 15-lipoxygenase gives values of about 20–30 turnovers per second and it represents the most active enzyme reported among the mammalian lipoxygenases that have been purified (35, 36). As the coral lipoxygenase domain has a mass of 79 kDa, fairly typical for animal (mammalian) lipoxygenases, whereas the soybean L-1 enzyme has a mass of 94 kDa, it is evident that the extra sequences in the plant enzyme do not represent the basis of its high catalytic activity. Nor does the fact that it is a 15-lipoxygenase. It is matched in activity in this case by a typically-sized animal 8R-lipoxygenase. The high activity of the coral 8R-lipoxygenase makes it well suited for partnership with the AOS domain, the turnover numbers of which are approximately 1400/s in the metabolism of 8R-HPETE. The results indicate that the catalase-related AOS enzyme can operate with similar efficiency to the plant AOS enzymes, which are P450 cytochromes (14, 15).

When 20.3o6 was used as substrate, an unexpected and mechanistically interesting reaction of the coral lipoxygenase domain involved the synthesis of small amounts of a 10-hydroperoxy derivative in addition to the main 8-hydroperoxide. There were indications of a very small trace of the corresponding product from arachidonic acid, but too little to permit identification or to be evident when viewed on the same scale as the major 8R-HPETE product (Fig. 5A). The 20.3o6 is a less optimal substrate than arachidonic acid, and its “misfit” in the active site is also evidenced by formation of a small percentage of 11-hydroxy-20.3o6 by-product (cf. Ref. 37). The formation of a bis-allylic hydroperoxy derivative has been reported before among lipoxygenases only with the manganese-containing lipoxygenase of the fungus Gaumannomyces graminis (38). Both the fungal and P. homomalla enzymes are R-lipoxygenases, and it remains to be seen if there is some structural element in the lipoxygenase-catalyzed biosynthesis of the R-hydroperoxides that favors hydrogen abstraction and oxygenation on the same carbon. It is also possible that the reaction occurs to a small extent in other lipoxygenases. The bis-allylic products undergo facile rearrangement under mildly acidic conditions to the more typical HETEs containing a conjugated diene (27, 39). As acid is used in typical fatty acid extraction procedures, synthesis of bis-allylic products can be easily missed.

A common feature of the catalysis by the LOX and AOS enzymes is their rapid loss of activity. The mechanisms are different in each case. The lipoxygenase loses activity simply upon dilution into the detergent-free buffer required for catalysis. Therefore, in conducting routine spectrophotometric assays using the absorbance increase at 235 nm, it was necessary to add the arachidonic acid substrate first, followed by enzyme to initiate reaction. Substantially reduced rates of reaction were observed when the additions were made the other way around. When the reaction rate was monitored at 235 nm, the enzyme maintained high activity for only 10–20 s. By 1 min at room temperature, the enzyme was almost completely inactivated.

Reports on the inactivation of soybean and porcine leukocyte lipoxygenases have correlated the production of secondary reaction products with their suicide inactivation (40–42). On observing reaction of the lipoxygenase domain of the coral fusion protein by repetitive scanning in the UV (200–350 nm), it was noticed that the absorbance changes showed only the formation of a conjugated diene, indicating no formation of mono-hydroperoxide. There was almost no associated formation of products having absorbances in the region of 270–280 nm (< 2% of the absorbance increase at 235 nm), indicating no significant formation of conjugated dienes and of the conjugated triene chromophore characteristic of double dioxygenation products and of derivatives of a leukotriene A type of epoxide. It appears that the loss of enzyme activity may be related to physical factors such as insolubility of the enzyme rather than a mechanism-based inactivation. By contrast, inactivation of the AOS domain is related to turnover. The high initial rate of reaction begins to decrease immediately and the enzyme is inactivated within 1 min. Use of a stopped flow spectrophotometer with fast kinetics was necessary to allow measurement of the initial reaction rate. Turnover-related inactivation is commonly observed among enzymes that synthesize or metabolize peroxide or epoxide substrates. For lipoxygenase and cyclooxygenase enzymes, the molecular basis of this inactivation has not been elucidated; oxidation of the protein is a primary suspect. Thromboxane synthase and leukotriene A₄ hydrolase show mechanism-based inactivation, and leukotri-
ene $A_2$ hydrolase has been shown to covalently bind a reaction intermediate (43, 44).

Our results show that the two domains of the unusual peroxidase-lipoxygenase fusion protein of $P. homomalla$ have activities well suited for the efficient conversion of arachidonic acid to the 8,9-epoxy allene oxide. It remains to be established how the two domains of the fusion protein interact together and whether the intermediate fatty acid hydroperoxide is specifically channeled to the AOS domain. It has been pointed out before that the presence of the peroxide-metabolizing AOS domain could interfere with activation of the lipoxygenase metabolism (45). This opens the question of how the lipoxygenase domain becomes activated in the face of a peroxide-metabolizing AOS domain. It is notable also that the mechanistic basis for the conversion of specific fatty acid hydroperoxides to allene oxides is completely uncharacterized either for the plant AOS cytochromes P450 or for the coral AOS domain. That the AOS domain has similarities in sequence to catalase yet distinctly different catalytic activity is an additional facet of the structure-function that will require an explanation of the role of individual amino acids in catalysis.

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