Purification and Molecular Cloning of an 8R-Lipoxygenase from the Coral Plexaura homomalla Reveal the Related Primary Structures of R- and S-Lipoxygenases*

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Lipoxygenases that form S configuration fatty acid hydroperoxides have been purified or cloned from plant and mammalian sources. Our objectives were to characterize one of the lipoxygenases with R stereospecificity, many of which are described in marine and freshwater invertebrates. Characterization of the primary structure of an R-specific enzyme should help provide a new perspective to consider the enzyme-substrate interactions that are the basis of the specificity of all lipoxygenases. We purified an 8R-lipoxygenase of the prostaglandin-containing coral Plexaura homomalla by cation and anion exchange chromatography. This yielded a colorless enzyme preparation, a band of ~100 kDa on SDS-polyacrylamide gel electrophoresis, and turnover numbers of 4000 min\(^{-1}\) of 8R-lipoxygenase activity in peak chromatographic fractions. The full-length cDNA was cloned by PCR using peptide sequence from the purified protein and by 5' and 3' rapid amplification of cDNA ends. The cDNA encodes a polypeptide of 715 amino acids, including over 70 amino acids identified by peptide microsequencing. A peptide sequence of 52 amino acids is cleaved to give the mature protein of 76 kDa; the difference from the estimated size by SDS-PAGE implies a post-translational modification of the P. homomalla enzyme. All of the iron-binding histidines of S-lipoxygenases are conserved in the 8R-lipoxygenase. However, the C-terminal amino acid is a threonine, as opposed to the isoleucine that provides the carboxylate ligand to the iron in all known S-lipoxygenases. These results establish that the 8R-lipoxygenase is related in primary structure to the S-lipoxygenases. A model of the basis of R and S stereospecificity is described.

Lipoxygenases are nonheme iron dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids to specific hydroperoxide products (1, 2). The enzymes occur widely in plants and animals, where they function in the biosynthesis of signaling molecules and bioactive mediators (1–4). Plant and mammalian lipoxygenases are related in primary structure and contain certain absolutely conserved amino acids that are critical for catalytic activity (5). From the crystal structures of the soybean lipoxygenases L-1 and L-2 it is known that three histidine residues and the C-terminal carboxyl of the protein (invariably an isoleucine) are ligands of the active site iron (6–9). One of the soybean lipoxygenase L-1 crystal structures has an additional iron ligand (7), represented as an asparagine in plants and an asparagine or histidine in the mammalian enzymes. These iron ligands are among the conserved residues of all lipoxygenases and, as indicated by site-directed mutagenesis, are essential to the function of these enzymes (10–12).

All the lipoxygenases characterized so far form hydroperoxides of the S stereoconfiguration. There exists, however, a group of enzymes in several species of invertebrate that catalyze the oxygenation of their polyunsaturated fatty acid substrates with R stereochemistry. These R lipoxygenases are reported in coral, sea urchin eggs, oocytes of starfish and clams,1 crabs, barnacles, and marine and freshwater hydroids (13–18, 20–23). They catalyze oxygenation in the 8R, 11R, or 12R configurations. These enzymes, in crude cell extracts, show the expected features of a typical lipoxygenase family member: (i) the primary products are specific hydroperoxides; (ii) the catalysis proceeds with an initial hydrogen abstraction (reflected in a strong primary isotope effect when the hydrogen is replaced with tritium) (17, 24); and (iii) there is an antarafacial relationship between the hydrogen abstraction and the insertion of molecular oxygen (17, 24). Notwithstanding this evidence, none of the R-specific enzymes have been purified, and there is no evidence to establish whether they constitute related family members or a completely different group of enzymes. The mammalian cyclooxygenases, as an example, can show similar catalytic features to lipoxygenases in crude extracts, yet these are hemoproteins and completely unrelated in structure.

One source of R-lipoxygenase, the coral Plexaura homomalla, has a distinguished history in the eicosanoid field. It contains 2–3% by weight of prostaglandin esters and for many years served as a commercial source of prostaglandins (25, 26). In 1987, we reported that extracts of P. homomalla avidly metabolize arachidonic acid to the 8R-hydroperoxide (8R-HPETE)2 (16). Based on preliminary investigations of the feasibility of purifying an R-lipoxygenase from a number of marine sources, we selected P. homomalla as a particularly rich source of enzyme. Here we report the purification, peptide microsequencing, and molecular cloning of a P. homomalla 8R-lipoxygenase. The results contribute toward our long term goal of an understanding of the basis of specificity of the S- and R-lipoxygenase enzymes.

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1 T. Hada and A. R. Brash, submitted for publication.

2 The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; RP-HPLC, reversed phase HPLC; SP-HPLC, straight phase HPLC; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); E911, Emulgen 911.
Experimental Procedures

Materials

Arachidonic acid was purchased from NuChek Prep Inc (Elysian, MN), and [1-14C]arachidonic acid was purchased from DuPont NEN. HETE standards were prepared by vitamin E-controlled autoxidation (27), and 8R-HETE was synthesized using the BR-lipoxygenase of P. homomalla (16). P. homomalla was collected in the Florida Keys and immediately placed on dry ice and stored at −80°C.

8R-Lipoxygenase Purification Protocol

The 8R-lipoxygenase activity was solubilized from 2 g of coral acetic acid powder (equivalent to about 10–20 g of coral) by stirring for 30 min at 4°C in 200 ml of 40 mM Tris, 2 mM EGTA, pH 8. The suspension was then centrifuged for 30 min at 10,000 x g at 4°C, the supernatant was collected, and the supernatant 911 (E911) digest was added to a final concentration of 0.1%. After stirring for 30 min at 4°C, the black-colored solution was loaded on an open bed Q-Sepharose column (10 ml of 25 cm, 50 ml bed volume) equilibrated in 40 mM Tris, 2 mM EGTA, pH 8, with 0.1% E911. The column was washed with loading buffer until the eluant was almost colorless (~200 ml), and then the lipoxygenase activity was eluted with the same buffer containing 0.4 M NaCl. Fractions of 10 ml were collected and assayed for lipoxygenase activity by measurement of the rate of increase in absorbance at 235 nm on conversion of arachidonic acid to 8R-HHETE; an aliquot of each fraction (50 μl) was diluted to 1 ml with 40 mM Tris, pH 8, and then arachidonic acid (10 μg in 2 μl of ethanol) was added, and the increase in absorbance at 235 nm was recorded for 1–2 min. Protein was assayed with the BCA (bicinchoninic acid) protein assay kit (Pierce) according to the standard curves of albumin in equivalent concentrations of E911 detergent).

Fractions with lipoxygenase activity were pooled (100–150 ml total) and dialyzed for 4–5 h with 2 liters of 2.5 mM sodium phosphate, pH 7, 0.1% E911 and then overnight using another 2 liters of fresh dialysis buffer. The sample was then loaded onto an open bed column of 10 ml of hydroxyapatite Bio-Gel-HT (Bio-Rad) and eluted with 100 mM sodium phosphate, pH 7, 0.1% E911. Fractions of 5 ml were collected, and active fractions were pooled (one or two fractions). Immediately prior to cation exchange chromatography, the sample was acidified to pH 5 by careful addition of 4 N phosphoric acid (−25 μl/μl of sample) and then injected on a Mono-S HR 5/5 column (Pharmacia Biotech Inc) equilibrated in 50 mM sodium phosphate (pH 5.0), 0.1% E911. The column was eluted with equilibration buffer for 15 min at a flow rate of 0.9 ml/min (pressure limitations), and then programmed using a linear gradient to 1.3 M NaCl, 50 mM sodium phosphate, 0.5% E911 over 45 min with on-line UV detection at 280 nm. Fractions of 1 ml were collected in tubes containing 0.1 ml of 1 M Tris pH 8, and aliquots were assayed for lipoxygenase using the UV assay. Fractions containing lipoxygenase activity were pooled and dialyzed overnight with 2 liters of 40 mM Tris (pH 8.0), 2 mM EGTA, and 0.1% E911.

The sample was then loaded on a Mono-Q HR 5/5 column (Pharmacia) equilibrated in 40 mM Tris, pH 8.0, 2 mM EGTA, and 0.1% E911. The column was eluted with equilibration buffer for 20 min at 0.75 ml/min and then programmed from 2% Tris to 0.4 M NaCl, 40 mM Tris, pH 8.0, 2 mM EGTA, and 0.3% E911. To remove the E911 detergent, active fractions were pooled, dialyzed into 2.5 mM sodium phosphate (pH 7), and loaded on a hydroxyapatite Biogel-HT column (5 × 0.5 cm, 1 ml). After washing with equilibration buffer (2.5 mM sodium phosphate) to remove the detergent, the buffer was changed to 200 mM sodium phosphate, and the active lipoxygenase was collected in 1–2 ml.

The 8R-lipoxygenase was further purified in denatured form by RP-HPLC on a C4 Vydac column (25 × 0.46 cm) using a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The proteins were detected by on-line UV monitoring at 214 nm, and aliquots of the UV-absorbing fractions were analyzed by SDS-PAGE to identify the ~100 kDa protein; it represented the main UV-absorbing peak by RP-HPLC. Semi-preparative SDS-PAGE was used as an alternative method for further purification of the ~100 kDa protein.

CNBr Cleavage/HPLC of Peptides

The purified 8R-lipoxygenase was taken to dryness and redissolved in 100 μl of 70% formic acid, and one or two crystals of cyanogen bromide were added. The reaction was allowed to proceed for 24 h in the dark at room temperature under nitrogen. Water (1 ml) was added, and the sample was taken to dryness under vacuum using a Speedvac (Savant). To eliminate poor chromatographic performance attributed to formylation of peptides (28), the sample was treated with 20 μl of ethanolamine for 5 min at room temperature and then evaporated to dryness. The peptides were then dissolved in 5 μl guanidine, 0.1 M Tris, pH 8.5, 0.1 mM diithiothreitol, warmed for 5 min at 50°C to reduce disulfides, and then separated by RP-HPLC using a C4 Vydac column (25 × 0.46 cm) and a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid.

HPLC Analysis of Lipoxygenase Metabolism

The lipoxygenase metabolism of [1-14C]arachidonic acid was evaluated essentially as described previously (16, 24). Following incubation with substrate (50 μM or 100 μM [1-14C]arachidonic acid), products were extracted using the Bligh and Dyer procedure (29), and the extracts were analyzed by RP-HPLC, SP-HPLC, and chiral column analysis. Prior to chiral column analysis, an aliquot of the sample was run on RP-HPLC, and then the main sample was injected and the products were collected. Following methylation with diazomethane and treatment with triphenylphosphine to reduce the hydroperoxides, the HETE methyl ester was collected on SP-HPLC, and then the stereochemistry was analyzed using a Chiralcel OD column (30).

Quantitation and HPLC of PCR Primers

The molar extinction coefficient of primers was calculated (31), the concentration was established by UV spectroscopy, and the working solutions were prepared at 4 μM. For PCR reactions involving the production of full-length lipoxygenase cDNAs, the PCR primers were ordered with the dimethoxytrityl protecting group on. The dimethoxytrityl protected primers were purified by RP-HPLC using a Hamilton 5-μm PRP-1 column (15 × 0.41 cm) run at a 0.7 ml/min flow rate using an initial solvent of acetonitrile, 0.1 M TEAA buffer (triethylamine acetate, pH 6.5) (95:5, v/v) and programmed to acetonitrile, 0.1 M TEAA buffer in the proportions 40:60 (v/v) over 40 min at a flow rate of 0.7 ml/min. The failed sequence products (which lack a dimethoxytrityl group) eluted at 18–22 min, and the desired product, a single main peak, eluted at 35–37 min. The main peak was collected, evaporated to approximately half volume, and then deprotected on a reversed-phase C18 Poly-Pak resin (Glen Research, Sterling, VA) by treatment on column with 2% trifluoroacetic acid according to the manufacturer’s instructions. The deprotected primer was eluted with water/acetonitrile (80:20, v/v), evaporated to near dryness to remove the acetonitrile, and quantified by UV spectroscopy.

Preparation of Total RNA

In our experience it is very difficult to obtain clean RNA from P. homomalla. Simple extractions such as the Chomczynski and Sacchi method (32), or the CsCl method of Chirgwin et al. (33) give brown-colored pellets that cannot be converted to cDNA. The following procedure was developed by a slight modification of methods designed for RNA preparation from “difficult” sources, bark of yew tree (of interest for taxol synthesis) (34) and marine algae (35); it gave an almost colorless pellet of RNA that was used successfully for cDNA synthesis. Approximately 10 g of P. homomalla was stored at −80°C was pulverized to a fine powder in liquid nitrogen. The pulverized coral was immediately homogenized in 30 ml of lysis buffer (150 mM Tris borate, pH 7.5, 2% SDS, 1% β-mercaptoethanol, 50 mM EDTA, with 0.4 g of polyvinylpyrrolidone (Sigma)) using four short full-speed bursts with a Polytron. Absolute ethanol (¼ volume) and 5 μl potassium acetate (¼ volume) were added, and the mixture was vortexed for 1 min. The solution was then extracted with 1 volume of chloroform/mixed isoamyl alcohol (49:1, v/v), and the phases were separated by centrifugation at 5000 x g for 10 min. The aqueous (top) phase was collected and reextracted with one volume of phenol/chloroform/mixed isoamyl alcohol (24:2:1, by volume, and the sample was then centrifuged again at 5000 x g for 10 min. The top phase was collected. Precipitation of the total RNA was carried out by addition of ¼ volume of 12 M LiCl and β-mercaptoethanol (final concentration of 1%, v/v) at −20°C for 48 h. The total RNA was pelleted by centrifugation at 20,000 × g for 90 min and resuspended with 4 ml of cesium chloride (400 mg/ml). Further purification of the P. homomalla total RNA was carried out by layering the sample on a cushion of cesium chloride and centrifugation at 31,000 rpm using a SW 41 rotor for 20 h at 10°C (34). The pellet of RNA was dissolved in water (0.5 ml) and quantified by UV spectroscopy. Approximately 100 μg of total RNA is recovered using this protocol.

cDNA Synthesis

cDNA reactions were run as described previously (36) using either random hexamer primers or an oligo-dT sequence linked at the 5′-end to an adaptor sequence (5′-ATG-AAT-TCC-GTA-CCC-GG-GATC-CT(T)17-3′); cDNA synthesis for 5′-RACE is described in the next section.
Primary Structure of 8R-Lipoxygenase

PCR Experiments

Initial PCR Clone—Upstream degenerate primers were based on the peptide sequences GPAPAKETI, 5′-GGI-TTY-CCG-AAR-ATI-GAR-ACI-AT′-3′ (where i is inosine, R is A or G, and Y is C or T), and for the first round nested PCR reaction (E); DILIDV, 5′-AR-GAY-ATI-ATT-GAY-GT-AT-3′. Downstream primers were based on conserved sequences at the C terminus of plant lipoxygenases (discussed later): NSISi-stop, 5′-ACC-TCA-GAT-GGA-GAT-RCT-RTT-3′, and for the second round nested PCR reaction, GIPNS, 5′-AT-RTT-RCT-DAT-ICC′-3′ (where D is A, G, or T). We noticed that the first of the two serines in these downstream sequences invariably is encoded by AGY (in sense code) in all known plant and mammalian lipoxygenases, thus serines in these downstream sequences invariably is encoded by AGY (in sense code). We ordered a second round of PCR reaction was primed with P. homomalla cDNA prepared from 1 μg of total RNA/50-μl PCR reaction and using 10 μl Tris, pH 8.3, 50 mM KCl, 3 mM MgCl2, with 0.2 μl of each dNTP and 0.25 μl (1.25 units) of AmpliTaq DNA polymerase (Perkin-Elmer) in a Perkin-Elmer 480 thermocycler. After the addition of the cDNA at 80°C (hot start), the PCR was programmed in a touchdown protocol (37) as follows: 94°C for 2 min, 1 cycle; 55°C for 1 min, 72°C for 1 min, 3 cycles; this was repeated in another 3 cycles, except with the annealing temperature lowered by 1°C to 54°C, and so on in 1°C increments until reaching 45°C (a total of 33 cycles). The protocol was completed with one cycle of 72°C for 10 min, and then the block temperature was held at 4°C. The second round reaction was primed with the encoding of the first of the two serines in the downstream production (adaptors), and this apparently facilitated annealing of the primer and the original downstream primer (ANVGGV); after a hot start at 80°C by spiking with the equivalent of 0.1 enzyme units of AmpliTaq DNA polymerase (Perkin-Elmer) in a Perkin-Elmer 480 thermocycler. After the addition of the cDNA at 80°C (hot start), the PCR was programmed in a touchdown protocol (37) as follows: 94°C for 2 min, 1 cycle; 60°C for 1 min, 72°C for 30 s, 94°C for 1 min, 3 cycles; this was repeated in another 3 cycles, except with the annealing temperature lowered by 1°C to 59°C, and so on in 1°C increments until reaching 50°C (and a total of 33 cycles). The protocol was completed with one cycle of 72°C for 10 min, and then the block temperature was held at 4°C. The second round PCR reaction used the PKEEPGD upstream primer and the original downstream primer (ANVGGV); after a hot start at 80°C by spiking with the equivalent of 0.1 μl of the first round PCR products, the PCR was programmed as follows: 94°C for 2 min, 1 cycle; 55°C for 1 min, 72°C for 30 s, 94°C for 1 min, 30 cycles; 72°C for 10 min, and then the block temperature was held at 4°C. Additional Internal Sequence—The sequence between the N-terminal and C-terminal boxes was sequenced using PCR by using random primed cDNA and the upstream primer 5′-ACG-GAA-ATG-AGA-GGA-TCA-CGA-G3′ and the downstream primer 5′-CAC-AGG-ATA-GTT-GAT-AGC-GTG-3′. The PCR protocol was based on 94°C, 2 min, 1 cycle, and then 30 cycles of 62°C for 1 min, 72°C for 1 min 30 s, 94°C for 1 min and completed by a 10-min extension at 72°C, and then the block temperature was held at 4°C.

3′-RACE—This was achieved using 5′-strand cDNA prepared using the adaptor-linked oligo(dT) primer. The upstream primer for the first round PCR was 5′-AAC-CGA-ATT-GTA-TTT-ATT-CCT-3′, and the downstream primer was the adaptor sequence 5′-TTG-CGC-TCC-CC-3′ with a PCR program based on 94°C, 2 min, 1 cycle and then 30 cycles of 55°C for 1 min, 72°C for 1 min, 30 s, 94°C for 1 min and completed by a 10-min extension at 72°C, and then the block temperature was held at 4°C.

5′-RACE Giving an ~450-bp PCR Product—This was accomplished using the Marathon cDNA amplification kit (Clontech). We modified the first strand cDNA synthesis by using 5 μg of total RNA (1 μg of mRNA or total RNA is recommended) and otherwise followed the manufacturer’s instructions and guidelines. The final preparation of double-stranded cDNA ligated 5′ and 3′ with adaptor sequences was diluted to 100 μl, and 5 μl was used per 50-μl PCR reaction. For the 5′-RACE reactions, the upstream primers for the first and second round reactions were specific for the ligated adaptor sequence, 5′-CACCTCCTAATAC-GACTCATTATAGGCGGG-3′ and 5′-ACTACATATAGGCGGCTGCACCAGG-3′, respectively. The downstream primer for the first round reaction was immediately downstream of the most 3′ conserved histidine, 5′-CAC-AGG-ATA-GTT-GAT-AGC-GTG-3′. The PCR program was 94°C for 2 min, 1 cycle; 68°C for 3 min, 94°C for 30 s, 30 cycles; 68°C for 7 min, 1 cycle; hold at 4°C. For the second round reaction (primed with 5′μl of the first round products) the downstream primer was selected based on cDNA sequence about 300 bp downstream of the protein N terminus, 5′-TGG-GGC-GGT-GTA-AGC-TTA-3′, and the PCR protocol was 94°C for 2 min, 1 cycle; 62°C for 30 s, 68°C for 2 min, 94°C for 30 s, 30 cycles; 68°C for 7 min, 1 cycle; hold at 4°C.

Full-length Clones Obtained by PCR—The upstream primer encoded the N terminus of the mature protein, with an added methionine codon and Koak consensus sequence for translation initiation (38) and a BamHI site added at the 5′-end to facilitate subcloning: 5′-AC-GCA-TCC-ACC-ATT-GTT-GAT-AGC-GTG-CTG-ATG-3′. The PCR program was 94°C for 2 min, 1 cycle; 65°C for 30 s, 68°C for 2 min, 94°C for 30 s, 30 cycles; 68°C for 7 min, 1 cycle; hold at 4°C.

DNA Sequencing

PCR products were subcloned into the pCRII vector (Invitrogen) and sequenced using Sequenase 2.0 (U.S. Biochemical Corp./Amersham Corp.), adenosine (ω-3S)-thiotriphosphate, and the dideoxy chain termination method.

RESULTS

Purification of the 8R-Lipoxygenase

Isolation of Active Lipoxygenase—This procedure is outlined in Fig. 1. Initial preparation of an acetone powder of P. homomalla removes the endogenous prostaglandins and other lipophilic components and also serves to reduce the salt content of the coral extract. The lipoxygenase activity dissolves in cold pH 8 buffer (no detergent is required) and is recovered in the 100,000× g pellet of the coral extract. The lipoxygenase activity dissolves in cold pH 8 buffer (no detergent is required) and is recovered in the 100,000× g pellet of the coral extract. The lipoxygenase activity dissolves in cold pH 8 buffer (no detergent is required) and is recovered in the 100,000× g pellet of the coral extract. The lipoxygenase activity dissolves in cold pH 8 buffer (no detergent is required) and is recovered in the 100,000× g pellet of the coral extract.
1. Prepare Acetone Powder of *P. homomalla*
2. Extract with 40 mM Tris + 2 mM EGTA, pH 8
3. Q-Sepharose (open bed) - retention black contaminants
   - elute with 0.4 M NaCl + 0.3% E911
   - dialyze with 2.5 mM Na phosphate (pH 7)
4. Hydroxyapatite-Biogel-HT (open bed)
   - elute with 100 mM Na phosphate + 0.1% E911
   - just before loading onto Mono-S
   - add dill H$_2$PO$_4$ (2.5 μl per ml)
5. Mono-S (FPLC, ion exchange)
   - elute with gradient to 1.3 M NaCl + 0.5% E911
   - dialyze with 40 mM Tris/2mM EGTA, pH 8
6. Mono-Q (FPLC, anion exchange)
   - elute with gradient to 0.4 M NaCl + 0.3% E911
   - dialyze with 2.5 mM Na phosphate, pH 7
7. Hydroxyapatite-Biogel-HT - removes detergent
   - wash with 2.5 mM Na phosphate
   - elute with 200 mM Na phosphate
8. C4 Vydac Reversed-phase HPLC
   or
   Preparative SDS-PAGE

**Fig. 1. Outline of the purification scheme for *P. homomalla* 8R-lipoxygenase.**

Purification of the active 8R-lipoxygenase (Fig. 2B). When fractions are collected across this UV peak and aliquots are examined by SDS-PAGE, it is apparent that the intensity of a protein band of ~100–105 kDa closely parallels the activity of the 8R-lipoxygenase (Fig. 2B, inset). The molecular masses of known lipoxygenases are approximately 75 kDa for the mammalian enzymes and 94–103 kDa for plant lipoxygenases (2, 39, 40).

Activity of Purified Protein—At this stage in the purification, the ~100-kDa component accounts for at least half of the protein in the sample (Fig. 3). The specific activity of the 8R-lipoxygenase is enriched approximately 30-fold compared with the Q-Sepharose eluant (protein concentration could not be measured in the initial black extract) (Table 1). This enzyme preparation forms 8-HPETE product solely of the 8R configuration as shown by chiral column analysis of the corresponding HETE methyl ester (Fig. 4). The most concentrated solutions of 8R-lipoxygenase (~0.1 mg/ml protein) were colorless and gave a featureless UV-visible spectrum. The addition of heme had no effect on activity. The turnover number of the purified sample is (in the peak fractions) approximately 4000 min$^{-1}$, compared with reported values for pure mammalian lipoxygenases of 5000–6000 min$^{-1}$ (e.g. Refs. 41 and 42) and ~20,000 for the soybean lipoxygenase L-1 (1). These results confirm that the lipoxygenase constitutes a major component of the purified extract.

Lipoxygenase Homology—The ~100-kDa band was retrieved in denatured form by RP-HPLC on a C4 Vydac column using a water/acetonitrile, 0.1% trifluoroacetic acid system or, in some experiments, by preparative SDS-PAGE. The amino acid composition closely matched that reported for the soybean lipoxygenase L-1 and mammalian lipoxygenases (Fig. 5). The N-terminal sequence did not match other lipoxygenases, but a BLAST search on an internal peptide recovered following CNBr cleavage gave a match to the soybean lipoxygenase L-1, and significant homology to the same region of mammalian enzymes was evident (Fig. 6). These results provided the first evidence of the closely related primary structure of S- and R-lipoxygenases.

**Fig. 2. Purification of the 8R-lipoxygenase by ion exchange chromatography.** Panel A, chromatography on a Mono-S HR 5/5 cation exchange column. Initially the column is equilibrated in 50 mM sodium phosphate, pH 5.0, and programmed with a linear gradient from 15 to 60 min to 1.3 mM NaCl, 50 mM sodium phosphate, 0.5% E911, pH 5.0. Aliquots of each fraction are assayed for lipoxygenase activity with a UV assay. Details of the procedures are given under “Experimental Procedures.” Panel B, chromatography on a Mono-Q HR 5/5 anion exchange column. Initially the column is equilibrated in 40 mM Tris, pH 8.0, 2 mM EGTA, and 0.1% E911 and then programmed with a linear gradient from 20 to 60 min to 0.4 mM NaCl, 40 mM Tris, pH 8, 2 mM EGTA, and 0.3% E911. Aliquots were analyzed for lipoxygenase with the UV assay. Details of the procedures are given under “Experimental Procedures.” Panel C, separate aliquots of the fractions covering the peak of lipoxygenase activity were analyzed by SDS-PAGE.

**Molecular Cloning of the 8R-Lipoxygenase**

Strategy for PCR Experiments—At first we were limited in the available peptide sequence to be used in molecular cloning.
The main peptide fragments obtained by CNBr cleavage did not chromatograph well on RP-HPLC, and the main peaks were mixtures of three peptides as determined by Edman microsequencing. We also obtained mixed sequences when the CNBr fragments were run on a high percentage acrylamide gel. The main peptide fragments obtained by CNBr cleavage did not result in a pure sequence because of the presence of artifacts in the electrophoresis. However, the CNBr fragments were used to design degenerate primers encoding the protein N terminus, which were used together with degenerate primers encoding the middle of the coding sequence to clone a 780-bp PCR fragment. This allowed the use of a fresh set of degenerate PCR primers, one of which was based on the CNBr peptide sequence on the first three or four residues into the protein. The plant lipoxygenase C terminus (see "Experimental Procedures") was designed based on conserved elements of the C-terminal sequence of the protein. Also, "guessmers" were designed based on conserved sequences of plant lipoxygenases. The plant and mammalian enzymes share an identical C-terminal isoleucine residue (a glutamine.

The enzyme activity is measured in a 1-ml reaction volume as absorbance units (AU) increase at 235 nm per min in an aliquot of the sample. In this table, the Mono-Q atep, 352 AU/min/mg for the whole sample corresponds to a turnover number of 1160 min⁻¹.

The initial 406-bp clone contained 406 bp in size. Sequencing of this fragment showed the conserved histidine and nearby asparagine residues corresponding to the most downstream of the characteristic iron ligands of the lipoxygenases, in addition to other conserved sequences.

Additional PCR Clones—The initial 406-bp clone contained one of the CNBr peptides that comprised a mixture of three peptides by Edman microsequencing. With this sequence revealed by cDNA cloning, and the second corresponding to the protein N terminus, we could deduce the third peptide sequence (MRGSRAPI... ) by a simple process of elimination. The peptide sequence was then cloned as a 780-bp PCR.
Product using gene-specific primers. The remaining 5'-sequence was obtained by 5'-RACE, and the remaining 3'-end of the cDNA sequence was readily cloned by 3'-RACE (see "Experimental Procedures").

Obtaining the Full-length Clone—cDNAs encoding the mature 8R-lipoxygenase protein were obtained by PCR using a proofreading mixture of Taq and Pwo DNA polymerases (see "Experimental Procedures"). One cDNA had the protein C terminus encoded in the downstream primer, and a second differed in using the 3'-untranslated region as downstream primer. The second product, therefore, did not predetermine the C-terminal cDNA sequence in the primer. This latter cDNA and the clones obtained by 3'-RACE had identical sequences at the C terminus, each showing that the C-terminal amino acid of the 8R-lipoxygenase is threonine (Fig. 7). The open reading frame encodes 715 amino acids. A signal peptide of 52 amino acids, all of which are in agreement with the predicted sequence from the cDNA. Since not one of the individual peptides codes for a different protein, the extensive match of amino acids shown in the CNBr peptides matches other lipoxygenases, and one of the CNBr peptides showed homology to the soybean lipoxygenase and the mammalian enzymes. The relationship was confirmed by molecular cloning of the cDNA, allowing firm assignment of the 8R-lipoxygenase to the same gene family as the known S-lipoxygenases.

It is the location of key amino acids in precisely the correct positions that establishes the relatedness of the 8R-lipoxygenase. The overall sequence identity of the P. homomalla 8R-lipoxygenase to other lipoxygenases is low, on the order of 15–20% in amino acid identity. However, the percentage of identity is higher (25–30%), and many conservative substitutions of amino acids are evident in the parts of the enzyme encompassing the iron-binding ligands. All of the absolutely conserved histidine residues of S-lipoxygenases are present in the 8R-lipoxygenase in the correct positions. Three of these histidines are ligands to the nonheme iron at the active site (6–9). The fifth heme ligand seen in one crystal structure of the soybean lipoxygenase (7), an asparagine, is also present in exactly the correct position in the 8R-lipoxygenase. Over 70% of the conserved residues of the S-lipoxygenases are retained in the P. homomalla enzyme (Fig. 8).

Features of the 8R-Lipoxygenase Protein—Our initial attempts at purification of the P. homomalla 8R-lipoxygenase were foiled by the strong tendency of the protein to chromatograph in protein microaggregates. It was only after development of the pH 5 cation exchange procedure that a significant enrichment in specific activity could be achieved. This low pH step seemed to "break" the microaggregates, something that could not be accomplished using detergents alone, and allow separation of the 8R-lipoxygenase. Purification yielded a protein that runs on SDS-PAGE with an apparent molecular mass of 100–105 kDa. This appeared compatible with the size of plant lipoxygenases, which have molecular masses of 94–103 kDa (39, 40). Based on the cDNA, however, the predicted size of the mature coral 8R-lipoxygenase protein is 76 kDa. This is typical of the only other class of animal lipoxygenase to be characterized, the mammalian enzymes. The presence of a prescence on the enzyme is unusual among lipoxygenases, to our knowledge reported only in the plant Lox2 genes exemplified by the inducible lipoxygenases of Arabidopsis and rice (40, 46).

The basis of the extra size estimated by SDS-PAGE has not been examined directly, although there are several reasons to deduce that it is related to post-translational modifications of the protein. High level expression of the mature protein in bacteria shows a strong protein band of 90 kDa (data not shown), corresponding more closely to the size anticipated from the cDNA. Post-translational modifications such as glycosylation might also account for the poor chromatographic characteristics of the natural P. homomalla protein. There are several potential sites for modification on the protein; for example, there are four asparagines with the NXT(S/T) consensus sequence for N-glycosylation, there are eight protein kinase C phosphorylation sites, and there are four possible sites for myristoylation (not shown).

An alternative explanation for the discrepancy in size observed by SDS-PAGE and molecular cloning is that the cDNA encodes a different protein. The extensive match of amino acids from peptide microsequencing argues strongly against this possibility. The established peptide sequence covers parts of the enzyme from the N terminus of the mature protein to near the carboxyl tail of the polypeptide. This includes over 70 amino acids, all of which are in agreement with the predicted sequence from the cDNA. Since not one of the individual peptides gives a match to another protein in the Swiss-Prot protein sequence data base, we can be confident that the match in over 70 amino acids indicates that we have cloned the correct cDNA.

Differences from Other Lipoxygenases—One of the striking features of the P. homomalla 8R-lipoxygenase is the presence of a threonine residue at the C terminus. This is unique among reported lipoxygenase sequences, all of which have an isoleucine in this position. We know from the crystal structure of the soybean lipoxygenases that the carboxyl of this isoleucine is a ligand to the active site iron (6–9), and site-directed mutagenesis studies confirm the importance of this residue. Using a murine 12S-lipoxygenase as the model enzyme, Chen et al. (47) showed that deletion of the C-terminal isoleucine yields a catalytically inactive protein. Substitution with valine was well tolerated, while only 10–20% activity was retained on changing to leucine, asparagine, or serine, less than 2% with arginine and glycine, and no activity on substitution with lysine or aspartate. A threonine substitution was not tested (47) and clearly is an inviting target for mutagenesis studies in the P. homomalla enzyme; the histidine in the second to last position is also of interest as a potential iron ligand. However, we have yet to develop a system for expression of active enzyme. We
were unsuccessful using our standard transient expression system, human embryonic kidney 293 cells. The explanation could be PCR-induced mutations in the cDNA, the need for post-translational modification, or, another possibility, the fact that the human embryonic kidney cells are incubated at 37°C and that this is unsuitable for expression of the \( \text{P. homomalla} \) \( 8\text{R} \)-lipoxygenase. Development of a prokaryotic expression system will be used to address this issue.

Based on the information available from this study alone, it is not possible to identify individual residues or sequences that constitute the structural basis for \( R \) and \( S \) stereospecificity among lipoxygenases. The \( 8\text{R} \)-lipoxygenase has less than 20% identity to the other enzymes; therefore, it is not possible to define the essential differences. We have available about 40 \( S \)-lipoxygenase sequences from animals and plants, and more \( R \)-lipoxygenase sequences are required for comparison.

**Primary Structure of 8R-Lipoxygenase**

![Fig. 7. Nucleotide sequence of the \( \text{P. homomalla} \) cDNA and the deduced amino acid sequence of the enzyme. Two PCR products encoding the mature 8R-lipoxygenase protein (FLGWL ... NSIHT) were fully sequenced (see “Experimental Procedures”). The addition sequence shown here from the 5\( \text{9} \)-end of the cDNA to the N terminus of the mature protein was obtained by 5\( \text{9} \)-RACE and the 3\( \text{9} \)-untranslated region by 3\( \text{9} \)-RACE. N-terminal and peptide sequence obtained by microsequencing of the purified protein is underlined. The peptide presequence (amino acids 1–52), which is not present in the mature protein, is shown in boldface type. The two fully sequenced clones of the mature protein had five single nucleotide differences that changed the encoded amino acid: at nucleotide position 575, A or G (Lys or Arg); at position 773, G or A (Gly or Asp); at position 820, A or G (Arg or Gly); at position 1603, G or T (Ala or Ser); at position 1634, C or A (Ala or Glu). Sequencing of these regions of an additional eight full-length clones showed all eight encoded A (Lys) at nucleotide position 575, G (Asp) at 773, A (Arg) at 820, T (Ser) at 1603, and G (Glu) at 1634. The consensus sequence is shown. Additionally, seven of the extra eight clones had a change of A to G (Asn to Asp) at nucleotide position 739.
Sequence alignment of representative plant and animal lipoxygenase with the P. homomalla 8R-lipoxygenase. The soybean L-1 enzyme and the human 12S-lipoxygenase are shown as representative of plant and animal lipoxygenases, respectively. The amino acids shown in these first two lines are the conserved amino acids in all animal and plant S-lipoxygenases, (with the exception of a sequence from the algae Porphyra purpura (45), which itself has fourteen changes to this consensus and was left out of this analysis). Each nonconserved amino acid is indicated by a * (or the number of nonconserved residues is given in parentheses). The iron ligands are in boldface type. On the third line, the corresponding amino acid of the P. homomalla 8R-lipoxygenase is shown (whether conserved or changed).

In this line of thinking (Fig. 9), there is a relation between 8R and 12S oxygenation, and similarly between that of 8S and 12R lipoxygenase catalyses to form either 8R- or 12S-HETEs involves an identical initial step, removal of the pro-S hydrogen from carbon 10 (Fig. 9, top). Reaction with oxygen occurs according to the well established antarafacial rule (i.e. oxygenation occurs on the opposite face of the substrate from the initial hydrogen abstraction). Reaction at one end of the original 1,4-cis,cis-pentadiene gives 8R-HETE, while reaction at the 12-carbon forms 12S-HETE. How the position of the reaction with O₂ is controlled has yet to be established for any lipoxygenase. Catalysis to form 8S or 12R products follows the same principles except that binding of substrate is in the reverse orientation (Fig. 9, bottom). The concept that substrate can bind one way round or the other is well preceded in the lipoxygenase literature. It is the most straightforward explanation for the formation of 9S-hydroperoxylinoleic and 13S-hydroperoxylinoleic acids by a single lipoxygenase enzyme (48, 49) and also for the 5S- and 8S-oxygenation activity of 15S-lipoxygenases (50).

This concept of the basis of R and S stereospecificity is supported by two examples of the formation of R configuration products by primarily S-lipoxygenases (51, 52). One of these cases involves the type 2 lipoxygenases of soybeans and peas, which, at pH 9, accept linoleic acid into a mixture of 13S-hydroperoxide together with 9-hydroperoxide composed of a 2-4-fold excess of the 9R enantiomer (51). The other example is a 12S-lipoxygenase of fish gills; it forms S configuration products in the first oxygenation, but it converts 15S-H(P)ETE to 8R,15S-DiH(P)ETE (52). Presumably, these enzymes have only one access channel for fatty acid into the active site, yet some R-configuration products can be formed. There is also a parallel to these concepts in the formation of the prostaglandin endoperoxide PGG₂ by the mammalian cylooxygenases (53, 54). This reaction involves two oxygenations of the fatty acid substrate, the first in the 11R configuration and the second in the 15S configuration. This is a double oxygenation parallel in many respects to the individual 8R and 12S oxygenations illustrated in Fig. 9. We conclude that R and S oxygenations in lipoxygenases involve different "fits" of the substrate and control of oxygenation and not a "mirror image" reaction at unre-
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