Manganese Lipoxygenase

DISCOVERY OF A BIS-ALLYLIC HYDROPEROXIDE AS PRODUCT AND INTERMEDIATE IN A LIPOXYGENASE REACTION

Linoleic acid was incubated with manganese lipoxygenase (Mn-LO) from the fungus Gäumannomyces graminis. The product consisted of (13R)-hydroperoxy-(9Z,11E)-octadecadienoic acid ((13R)-HPOD) and a new hydroperoxide, (11S)-hydroperoxy-(9Z,12Z)-octadecadienoic acid ((11S)-HPOD). Incubation of (11R)-[2H]- and (11S)-[2H] lipoic acid with Mn-LO led to the formation of hydroperoxides that largely retained and lost, respectively, the deuterium label. Conversion of the (11S)-deuteriolinoleic acid was accompanied by a primary isotope effect, which manifested itself in a strongly reduced rate of formation of hydroperoxides and in a time-dependent accumulation of deuterium in the unconverted substrate. These experiments indicated that the initial step catalyzed by Mn-LO consisted of abstraction of the pro-S hydrogen of linoleic acid to produce a linoleyl radical. (11S)-HPOD was converted into (13R)-HPOD upon incubation with Mn-LO. The mechanism of this enzyme-catalyzed hydroperoxide rearrangement was studied in experiments carried out with 18O2 gas or 18O2-labeled hydroperoxides. Incubation of [11,18O2]-labeled HPOD with Mn-LO led to the formation of (13R)-HPOD, which retained 39–44% of the 18O label, whereas (11S)-HPOD incubated with Mn-LO under 18O2 produced (13R)-HPOD, which had incorporated 57% of 18O. Furthermore, analysis of the isotope content of (11S)-HPOD remaining unconverted in such incubations demonstrated that [11,18O2]-(11S)-HPOD suffered a time-dependent loss of 18O when exposed to Mn-LO, whereas (11S)-HPOD incorporated 18O when incubated with Mn-LO under 18O2. On the basis of these experiments, it was proposed that the conversion of (11S)-HPOD into (13R)-HPOD occurred in a non-concerted way by deoxygenation into a linoleyl radical. Subsequent reoxygenation of this intermediate by dioxygen attack at C-13 produced (13R)-HPOD, whereas attack at C-11 regenerated (11S)-HPOD. The hydroperoxide rearrangement occurred by oxygen rebound, although, as demonstrated by the 18O experiments, the oxygen molecule released from (11S)-HPOD exchanged with surrounding molecular oxygen prior to its reincorporation.

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Lipoxygenase-catalyzed dioxygenation of polyunsaturated fatty acids leads to the formation of reactive fatty acid hydroperoxides. Mammalian lipoxygenases can catalyze oxygenation at carbons 5, 8, 12, and 15 of their predominant substrate, i.e. arachidonic acid (1). Many plant lipoxygenases can also utilize arachidonic acid, although their most important substrates are the C-18 fatty acids linoleic acid and α-linolenic acid (2). Interest in lipoxygenases stems partly from the fact that fatty acid hydroperoxides can be further metabolized into biologically active oxylipins such as leukotrienes and jasmonates.

Lipoxygenases contain ferrous iron, which is oxidized into the ferric state by, e.g., hydroperoxides. The ferric form of lipoxygenases is catalytically active (3) and catalyzes the stereospecific abstraction of one hydrogen from the bis-allylic methylene group of the (1Z,4Z)-pentadiene structure of the substrate as the initial step (4). Attack by dioxygen at one of the terminal positions of the resulting pentadienyl radical results in the formation of a hydroperoxide having one pair of E/Z-conjugated double bonds. Studies of the regio- and stereochemistry of the steps occurring in the oxygenation of 8,11,14-eicosatrienoic acid by soybean lipoxygenase-1 revealed that the pro-S hydrogen was stereospecifically removed from C-13 and that dioxygen was regio- and stereospecifically inserted at C-15 to produce a (15S)-hydroperoxide (4). This finding, and results of similar studies carried out with linoleic acid (9S)-lipoxygenase from corn (5), arachidonic acid (12S)-lipoxygenases from human platelets (6) and a red alga (7), arachidonic acid (5S)-lipoxygenases from rat basophil leukemia cells and potato (8), and arachidonic acid (8S)-lipoxygenase from mouse epidermis (9) indicated the existence of an antarafacial relationship between hydrogen abstraction and oxygen insertion as a common feature of dioxygenations catalyzed by lipoxygenases. Interestingly, such a stereospecific relationship has also been found for the dioxygenation catalyzed by an “R” lipoxygenase, i.e. (12R)-lipoxygenase from sea urchin (10), as well as for dioxygenations catalyzed by prostaglandin endoperoxide synthases I (11) and II (12), and by ferrylmyoglobin (13). Mammalian and plant lipoxygenases so far studied catalyze production of hydroperoxides that have the “S” absolute configuration. In contrast, a number of marine invertebrates, such as starfish, sea urchin, and the coral Plexaura homomalla, express lipoxygenases, which catalyze formation of “R” hydroperoxides, as demonstrated by the configuration of hydroperoxides formed by oxygenation of arachidonic acid at the C-5, C-8, C-11, and C-12 positions. One of these enzymes, arachidonic acid (8R)-lipoxygenase, was recently cloned and sequenced (14).

Manganese lipoxygenase (Mn-LO)3 was purified from the
fungus *Gäumannomyces graminis* as described in the accompanying paper (15). The protein was found to be heavily glycosylated and to contain 0.5–1 atom of manganese per molecule. The molecular mass of the native protein (~135 kDa) was comparable to that of arachidonic acid (15S)-lipoygenase purified from the fungus *Saprolegnia parasitica* (145–150 kDa) (16). Interestingly, the molecular mass of the deglycosylated Mn-LO (~73 kDa) (15) was similar to those of mammalian lipoygenases. The present paper is concerned with the stereochemistry and mechanism of the Mn-LO-catalyzed dioxygenation. In the course of the work, it was unexpectedly found that the enzyme catalyzes bis-allylic oxygenation of polyunsaturated fatty acids. Thus, oxygenation of linoleic acid resulted in the formation of 11-hydroxyoctadecadienoic acid, a new member of the oxylipin family of compounds.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1-14C]Linoleic acid was purchased from NEN Life Science Products. Dilution with unlabeled material (Nu-Chek-Prep, Elysian, MN) followed by purification by SiO2 chromatography afforded the desired compound. The material had a specific radioactivity of 5.0 kBq/μmol. The resulting stereospecifically deuterated stearic acids as described in the accompanying paper (15) were prepared by lithium aluminum deuteride reduction of the 100% (11S)-[2H]linoleic acid (37.2% monodeuterated and 62.8% undeuterated molecules) and (11S)-[2H]linoleic acid (32.6% monodeuterated and 67.4% undeuterated molecules) were prepared by purification by SiO2 chromatography. The resulting stereospecifically deuterated stearic acids were as described in detail elsewhere (17, 18). The optical purities of the chiral 11-hydroxystearates were: (11R)-hydroxystearate (precursor of (11S)-[2H]linoleic acid), 98.3% R; (11S)-hydroxystearate (precursor of (11R)-[2H]linoleic acid), 95.4% S. (9S)-HPOD and (13S)-HPOD were prepared by incubation of linoleic acid with tomato lipoygenase (19) and soybean lipoygenase (20). The corresponding alcohols, (9S)-HOD and (13S)-HOD, were obtained from the hydroperoxides by reduction with sodium borohydride. (11R)-HOD and 11-ketolinoic acid were prepared by incubation of linoleic acid with an enzyme preparation of the red alga *Lithothamnion corallioides* (21). 13O, 99.4 atom %, was obtained from Isotec, Miamisburg, OH. Glutathione peroxidase, reduced glutathione, and to contain 0.5–1 atom of manganese per molecule.

**Chemical Methods**—Preparation and purification of 11-menthoxycarbonyl (MC) derivatives (23), and sterically hindered 9-HOD and 13-HOD (23) and of 11-HOD (21) were performed as indicated. Catalytic hydrogenation was performed with platinum catalyst (3 mg) using methanol (1 ml) as the solvent. Partial hydrogenation of the methyl ester of 11-HOD (25 μg) was carried out by stirring with 5% palladium-on-carbonate (5 mg) in ethyl acetate (3 ml) under hydrogen gas for 4 min (cf. Ref. 21). Oxidative ozonolysis was carried out as described (23) using an ozone generator (model T-12) purchased from TriO3 Industries, Fort Pierce, FL.

**Chromatographic and Instrumental Methods**—RP-HPLC was performed with a column of Nucleosil 100–5 C18 (250 × 4.6 mm) purchased from Macherey-Nagel (Duren, Germany). The solvent system used consisted of acetoniwater/methanol (60:40:9.02, v/v/v). Straight phase high performance liquid chromatography was carried out with a column of Nucleosil 50–5 (200 × 4.6 mm) and a solvent system of 2-propanol/hexane (1:99, v/v). The absorbance (217 nm) and radioactivity of high performance liquid chromatography effluents were determined on-line using a Spectrnonitor III ultraviolet detector (Laboratory Data Control, Riviera Beach, FL) and a liquid scintillation counter (NIS Systems, Tampa, FL), respectively. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. Retention times were converted into C-values using standards of saturated fatty acid methyl esters (24). GC-MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. LC-MS was performed as described in the accompanying paper (15). Ultraviolet absorption as a function of wavelength or time was recorded with a Hitachi (Tokyo, Japan) model U-2000 UV-visible spectrophotometer. Infrared spectrometry was carried out using a Perkin-Elmer model 1650 FT-IR spectrophotometer. Radioactivity was determined with a Packard Tri-Carb model 4450 liquid scintillation counter (Packard Instruments, Downer’s Grove, IL).

**RESULTS**

**Oxidation of Linoleic Acid by Manganese Lipoygenase**

**Isolation of Reaction Products of Linoleic Acid**—[1-14C]Linoleic acid (350 μg) was stirred for 30 min with Mn-LO (1.5 μg) in buffer A (2.1 ml) at 23 °C. Material isolated by extraction with diethyl ether was subjected to RP-HPLC radiochromatography. As seen in Fig. 1, three peaks of radioactivity appeared. The least polar material (29%; 81.1 ml of effluent) was identical to [1-14C]linoleic acid remaining unchanged. Compound A (12%; 14.1 ml of effluent) was intermediate in polarity to references of authentic (11S,11R)-HOD (12.1 ml of effluent) and (13S)-HOD (15.4 ml of effluent). Compound B (59%; 17.0 ml of effluent) cochromatographed with authentic (13S)-HPOD.

**Identification of Compound C**—Analysis of compound C by LC-MS demonstrated a peak (10.4 min effluent) giving prominent ions at m/z 311 (M + 1; loss of H), 295 (M + 17; loss of OH), and 293 (M + 1; loss of H plus H2O). When compound C was treated in D2O/CDC13 (1:1, v/v), the M + 1 ion shifted to m/z 312. An aliquot of compound C was used for determination of Mn-Lo activity in presence of Mn-Lo, Mn-LO-catalyzed dioxygenation of the methyl esters of 13-HOD (79.1%; 11.6 ml of effluent) and 9-HOD (2.9%; 14.7 ml of effluent) as judged by their chromatographic behaviors, UV spectrophotometry, and mass spectrometry. The two hydroxyoctadecadienoates were converted into their MC derivatives and subjected to oxidative ozonolysis.
Analysis of the esterified product by GLC demonstrated the formation of dimethyl azelate as well as the MC derivative of methyl 2-hydroxyheptanoate (R/S, 97.5:2.5) from the derivative of 13-HOD. The derivative of 9-HOD afforded the MC derivative of dimethyl 2-hydroxysebacate (R/S, 37.0:63.0). These experiments confirmed the results of the accompanying paper (15), which identified the major hydroperoxide produced from linoleic acid as (13R)-HPOD. In addition, the analyses showed that the Mn-LO-catalyzed oxygenation of linoleic acid resulted in small amounts of the following hydroperoxyoctadecadienoate isomers: (13S)-HPOD (2.4%), (9R)-HPOD (1.1%), and (9S)-HPOD (1.8%).

**Structure of Compound A**—The retention time of compound A upon LC-MS analysis was 8.3 min, and the mass spectrum showed prominent ions at M−1 (M = 1), loss of H, 296 (M − 17), loss of OH, and 293 (M − 1 + 18); loss of H plus H2O. This result indicated that compound A was a hydroperoxyoctadecadienoic acid (molecular weight, 312). The UV spectrum of compound A was featureless (Fig. 2) demonstrating the absence of e.g., conjugated double bonds. Treatment of compound A with a small amount of peroxycetic acid resulted in a rapid appearance of UV absorption bands at 259, 268, and 279 nm (Fig. 2). Analysis of the esterified product by GLC and GC-MS demonstrated the presence of geometrical isomers of methyl 8,10,12- and 9,11,13-octadecatrienoates as judged by their molecular weight (292) and retention times (C-19:00 (21%), C-19:13 (21%), C-19:42 (28%), and C-19:46 (30%).) The fact that a virtually identical mixture of geometrical isomers of methyl 8,10,12- and 9,11,13-octadecatrienoates was formed upon acid treatment of the methyl ester of (11R)-HOD (21) suggested that compound A was a derivative of linoleic acid substituted at the bis-allylic position (C-11). Treatment of compound A with sodium borohydride followed by esterification afforded the methyl ester of 11-HOD as judged by GLC and GC-MS. The retention time corresponded to C-19:41, a value identical to that observed for the Me3Si derivative of the methyl ester of authentic 11-HOD but different from the values recorded for the Me3Si derivatives of the methyl esters of (9E,12Z)-11-HOD and (9Z,12E)-11-HOD (C-19:48/C-19:56) and (9E,12E)-11-HOD (C-19:62). The mass spectrum of the reduced product obtained from compound A was identical to that of the Me3Si derivative of the methyl ester of 11-HOD and showed prominent ions at m/z 382 (M, 38%), 311 (M−17; loss of •(CH3)2CH=CH2, 32%), 253 (9%), and 225 [(CH=CH=O)(OSiMe3)CH=CH=CH2] (44%). Reduction of compound A with sodium borodeuteride afforded 11-HOD without significant incorporation of deuterium (less than 0.5%), thus excluding the possibility of a 11-keto group in compound A. Catalytic hydrogenation of compound A using platinum as catalyst resulted in the formation of a 1:1 mixture of 11-hydroxyoctadecanoate and 11-ketoctadecanoate as shown by GC-MS analysis using the authentic compounds as references. On the basis of these data, compound A was identified as a 11-hydroperoxy derivative of linoleic acid. Partial hydrogenation of compound A followed by oxidative ozonolysis performed on the MC derivative was used to determine the double bond positions and the configuration of C-11. Analysis of the esterified ozonolysis product by GLC and GC-MS demonstrated the presence of the MC derivatives of methyl (2S)-hydroxynonanoate (less than 2% of the (2R)-hydroxynonanoate; fragment originating in the MC derivative of methyl 11-hydroxy-9-octadecenoate) and of dimethyl (2R)-hydroxydodecane-1,12-dioate (less than 2% of the (2S)-hydroxydodecenadioate; fragment originating in the MC derivative of methyl 11-hydroxy-12-octadecenoate). This experiment thus established that the two double bonds of compound A were localized in the A4 and A12 positions, and that the absolute configuration of the alcohol group at C-11 was “S.” The data presented demonstrated that compound A was identical to (11S)-hydroperoxy-(9Z,12E)-octadecadienoic acid (11S)-HPOD).

**Conversion of (11S)-HPOD into (13R)-HPOD**

**Formation of (11S)-HPOD and (13R)-HPOD as a Function of Time and Substrate Concentration**—Linoleic acid was incu-
 Amounts of (13\textsuperscript{R})-HPOD corresponded to (13\textsuperscript{R})-HPOD produced from (13\textsuperscript{R})-HPOD (1.7%); the same was true for the formation of (13\textsuperscript{S})-HPOD (52\textsuperscript{R} pmol min\textsuperscript{–1}). As judged by RP-HPLC radiochromatography, (13\textsuperscript{S})-HPOD (15\textsuperscript{R}) was obtained from linoleic acid with Mn-LO, the (13\textsuperscript{S})-HPOD produced from (13\textsuperscript{S})-HPOD as (13\textsuperscript{S})-HPOD was based on its UV spectrum (solvent methanol; \(\lambda_{\text{max}} = 234\) nm), its conversion into 13-HOD upon reduction with sodium borohydride, its conversion into 13-hydroxyxostearate upon reduction followed by catalytic hydrogenation, and on results of its chemical degradation by oxidative ozonolysis. As was the case with (13\textsuperscript{R})-HPOD isolated following incubation of linoleic acid with Mn-LO, the (13\textsuperscript{R})-HPOD produced from (13\textsuperscript{S})-HPOD was accompanied by small amounts of 8-hydroperoxystearates, i.e. (9\textsuperscript{R})-HPOD (1.2%) and (9\textsuperscript{S})-HPOD (1.7%).

Conversion of (11\textsuperscript{S})-HPOD into (11\textsuperscript{R})-HPOD was conveniently monitored by recording the UV absorbance at 235 nm versus time (Fig. 4). At the concentrations used, the maximum rate of formation of (13\textsuperscript{R})-HPOD from (11\textsuperscript{S})-HPOD, i.e. 18.3 nmol min\textsuperscript{–1} \(\mu\)g\textsuperscript{–1}, was 50% greater than that of formation of (13\textsuperscript{S})-HPOD from linoleic acid, i.e. 12.1 nmol min\textsuperscript{–1} \(\mu\)g\textsuperscript{–1}. Interestingly, a slight lag phase was consistently observed when (11\textsuperscript{S})-HPOD was used as the substrate (Fig. 4).

Incubations in the Presence of Glutathione Peroxidase—Glutathione peroxidase and reduced glutathione catalyze reduction of fatty acid hydroperoxides into hydroxy acids. Trapping of hydroperoxides by this enzyme has been used in previous studies to confirm the existence of fatty acid hydroperoxides as intermediates in the biosynthesis of epoxy-hydroxy acids (25) and dihydroxy acids (26, 7). If the Mn-LO-catalyzed formation of (13\textsuperscript{R})-HPOD from linoleic acid took place by the sequence linoleic acid \(\rightarrow\) (11\textsuperscript{S})-HPOD \(\rightarrow\) (13\textsuperscript{R})-HPOD, inclusion of glu-
HPOD (3 or 5 mM nase (600 units) in 1 ml of buffer B was treated with (11S)-HPOD (52 μM) in buffer C (1.05 ml) was treated with Mn-LO (1.1 μg) at 23 °C. The absorbance at 235 nm was measured using a cuvette having 10 mm path length. The maximum rate of increase of the absorbance at 235 nm was 0.50 absorbance unit/min corresponding to a rate of formation of (13R)-HPOD equal to 18.3 nmol min⁻¹ μg⁻¹. B, linoleic acid (53 μM) in buffer C (1.05 ml) was treated with Mn-LO (1.1 μg) at 23 °C and the absorbance was measured as described in A. The maximum rate of increase of the absorbance at 235 nm was 0.33 absorbance unit/min corresponding to a rate of formation of (13R)-HPOD equal to 12.1 nmol min⁻¹ μg⁻¹.

Fig. 4. Time courses of formation of (13R)-HPOD from (11S)-HPOD (A) and linoleic acid (B). A, (11S)-HPOD (52 μM) in buffer C (1.05 ml) was treated with Mn-LO (1.1 μg) at 23 °C. The absorbance at 235 nm was measured using a cuvette having 10 mm path length. The maximum rate of increase of the absorbance at 235 nm was 0.50 absorbance unit/min corresponding to a rate of formation of (13R)-HPOD equal to 18.3 nmol min⁻¹ μg⁻¹. B, linoleic acid (53 μM) in buffer C (1.05 ml) was treated with Mn-LO (1.1 μg) at 23 °C and the absorbance was measured as described in A. The maximum rate of increase of the absorbance at 235 nm was 0.33 absorbance unit/min corresponding to a rate of formation of (13R)-HPOD equal to 12.1 nmol min⁻¹ μg⁻¹.

Isotope Experiments

*18O₂ Experiments*—The mechanism of formation of hydroperoxides catalyzed by Mn-LO was studied in experiments where linoleic acid, (11S)-HPOD, or (13R)-HPOD were incubated with Mn-LO in the presence of *18O₂*. The products were isolated by RP-HPLC (cf. Fig. 1), and aliquots were reduced with sodium borohydride, esterified, and analyzed by GC-MS as the Me₃Si derivatives. In other experiments, (11-[*18O₂*]R)-HPOD and (13-[*18O₂*]R)-HPOD were biosynthesized from linoleic acid and reincubated with Mn-LO under ambient air.

Incubation of linoleic acid under *18O₂* led to the formation of (11S)-HPOD and (13R)-HPOD that had incorporated *18O₂* (Table I). Incubations of [11-*18O₂*]R(11S)-HPOD with Mn-LO led to the formation of (13R)-HPOD that retained 39–44% of the *18O₂* label. In addition, 9-HPOD, a minor hydroperoxide of Mn-LO catalysis, was labeled at the same level (incubations 2 and 4, Table I). In another experiment, (11S)-HPOD was incubated with Mn-LO under *18O₂*. As seen (incubation 5, Table I), this led to the formation of (13R)-HPOD that had incorporated a significant amount of *18O₂*. Furthermore, analysis of the *18O* content of (11S)-HPOD remaining not converted in incubations of [11-*18O₂*]R(11S)-HPOD conducted under air, and in an incubation of (11S)-HPOD under an atmosphere of *18O₂*, demonstrated a time-dependent exchange of the hydroperoxide oxygen with O₂ (incubations 4 and 5, Table I). Such oxygen exchange was not observed when (13R)-HPOD was treated with Mn-LO (incubation 6, Table I).

**Incubations of Stereospecifically Deuterated Linoleic Acids**—(11S)-HPOD and (13R)-HPOD biosynthesized from (11R)-[²H] and (11S)-[²H] linoleic acids were isolated by RP-HPLC and their isotope content determined by GC-MS after reduction, esterification, and conversion into the Me₃Si derivatives. As
seen in Table II, hydroperoxides produced from (11R)-[2H]-
linoleic acid retained most of the deuterium label, whereas hy-
droperoxides generated from (11S)-[2H]-linoleic acid lost most of
the label. These results demonstrated that the hydrogen ab-
stracted from the C-11 methylene group by Mn-LO had the pro-S
configuration. The isotope contents of linoleic acid re-
maining unconverted in incubations of (11R)-[2H]- and (11S)-
[2H]-linoleic acids were also determined. As seen (Table II),
icubation of (11S)-[2H]-linoleic acid was accompanied by a
time-dependent enrichment of deuterium in unconverted lino-
leic acid. The presence of a kinetic isotope effect in the enzyme-
catalyzed abstraction of the (11S) deuterium indicated by this
experiment also manifested itself in the time course of forma-
tion of (13R)-HPOD from (11S)-[2H]-linoleic acid measured
spectrophotometrically. As seen in Fig. 6, production of (13R)-
HPOD from (11S)-[2H]-linoleic acid (32.6% deuterated and
67.4% undeuterated molecules) occurred by a biphasic time
course. This was explainable if it is assumed that undeuterated
linoleic acid present in the mixture incubated was mainly oxy-
genated in the early phase of the incubation (segment A in
Fig. 6), whereas the gradually accumulating deuterated sub-
strate was oxygenated at a much slower rate during the later
phase (segments B and C in Fig. 6). Estimates of the rates of
conversion of undeuterated and deuterated molecules could be
made from the slopes of segments A–C, i.e. 7.9 nmol min
\(^{-1}\) for segment A (roughly corresponding to oxygenation
of undeuterated linoleic acid), and 0.4–0.5 nmol min
\(^{-1}\) for
segments B and C (roughly corresponding to oxygenation of
deuterated linoleic acid). The magnitude of the kinetic isotope
effect estimated from these rates was \(k_{HD}/k_{D} = 15–22\). The
reported value of the isotope effect in the soybean lipoxygenase-
catalyzed oxygenation of linoleic acid deuterated at C-11
(94–95% dideuterated molecules) is \(k_{HD}/k_{D} = 8–9\) (27, 28); how-
ever, much larger values have been reported recently (29–31).

An interesting finding in the experiments with the stereospe-
cifically deuterated linoleic acids was the slightly different
deuterium contents of (11S)-HPOD and (13R)-HPOD biosyn-
thesized from (11R)-[2H]-linoleic acid (Table II). The optical
purity of the (11S)-hydroxyestearate used to prepare the (11R)-
[2H]-linoleic acid was 95.4% (18); therefore, the percentage re-
tentions of deuterium observed for (11S)-[2H]-HPOD and
(13R)-HPOD (98%) were somewhat higher and lower, respec-
tively, compared with the theoretical value (95%). A second-
ary isotope effect has been described for the oxygenation of
(10R)-[3H]arachidonic acid catalyzed by human platelet 12-
lipoxygenase (32), and it is tempting to speculate that the
slightly different extent of labeling of the two hydroperoxides
formed from (11R)-[2H]-linoleic acid in the presence of Mn-LO is
due to a secondary isotope effect in the conversion of [11-
2H](11S)-HPOD into [11-2H](13R)-HPOD (cf. Fig. 7).

**Table I**

| Incubation | Substrate | \(^{18}\)O content of substrate | Condition | Time of incubation\(^a\) | Product isolated\(^b\) | Product share | \(^{18}\)O content of products\(^c\) |
|------------|-----------|-----------------------------|-----------|------------------------|------------------|---------------|----------------|
| 1\(d\)     | Linoleic acid | 0 | \(^{18}\)O\(_2\) | 30 | 11(S)-HPOD | ND | 77.6 |
|            |           | 0 | \(^{18}\)O\(_2\) | 13(R)-HPOD | ND | 79.3 |
| 2          | [18O\(_2\)](11S)-HPOD | 77.6 | Air | 7 | 13(R)-HPOD | 98 | 30.1 (39) |
| 3          | Linoleic acid | 0 | \(^{18}\)O\(_2\) | 30 | 11(S)-HPOD | ND | 97.2 |
| 4          | [18O\(_2\)](11S)-HPOD | 97.2 | Air | 1.7 | 11(S)-HPOD | 70 | 91.8 (94) |
|            |           | 0 | \(^{18}\)O\(_2\) | 13(R)-HPOD | 30 | 42.3 (44) |
|            |           | 0 | \(^{18}\)O\(_2\) | 13(R)-HPOD | 44 | 84.1 (87) |
|            |           | 0 | \(^{18}\)O\(_2\) | 13(R)-HPOD | 56 | 41.4 (45) |
| 5          | (11S)-HPOD | 0 | \(^{18}\)O\(_2\) | 3 | 11(S)-HPOD | 76 | 5.8 |
| 6          | [18O\(_2\)](13R)-HPOD | 98.1 | Air | 5 | 13(R)-HPOD | 24 | 57.0 |

\(^a\) Incubations were performed at 23 °C using buffer A.
\(^b\) Products were isolated by RP-HPLC, reduced by treatment with sodium borohydride, esterified, and converted into their Me\(_3\)Si derivatives. The isotope content was determined by GC-MS operated in the selected ion monitoring mode.
\(^c\) The percentages of \(^{18}\)O\(_2\)-containing species are indicated. Numbers within parentheses indicate the percentage retention of \(^{18}\)O relative to the precursor compound.
\(^d\) In this incubation, an accidental leakage in the glass apparatus used for the \(^{18}\)O\(_2\) incubation resulted in an incomplete \(^{18}\)O\(_2\) labeling of the hydroperoxides biosynthesized from linoleic acid.
Bis-allylic Dioxygenation of Linoleic Acid

### Table II

| Substrate | Monodeuterated molecules in substrate | Time of incubation | Product isolated | Product share | Monodeuterated molecules in substrate |
|-----------|--------------------------------------|--------------------|------------------|---------------|-------------------------------------|
| (11R)-[2H]Linoleic acid | 37.2 | 3.5 | (11S)-HPOD | 6 | 36.5 (98) |
| | | | (13R)-HPOD | 21 | 32.0 (96) |
| | 11.5 | Linoleic acid | 73 | 41.7 (112) |
| | 16 | Linoleic acid | 23 | 42.0 (113) |
| | | Linoleic acid | 5 | 43.6 (117) |
| (11S)-[2H]Linoleic acid | 32.6 | 4.5 | (11S)-HPOD | 4 | 1.8 (5) |
| | | | (13R)-HPOD | 22 | 1.3 (4) |
| | 15 | Linoleic acid | 74 | 44.6 (137) |
| | 49 | Linoleic acid | 39 | 75.2 (231) |
| | | Linoleic acid | 16 | 95.4 (293) |

* a Incubations were performed at 23 °C using buffer A.
* b Products were isolated by RP-HPLC. Recovered linoleic acid was treated with diazomethane, whereas hydroperoxides were reduced, esterified, and converted into their Me₃Si derivatives. The isotope content was determined by GC-MS operated in the selected ion monitoring mode.
* c Numbers within parentheses indicate the percentage enrichment of [2H] relative to the deuterated linoleic acid incubated. In the case of (11S)-[2H]linoleic acid, the upper limit of relative enrichment is 100 × (100/32.6) = 307%.

Fig. 6. Rates of formation of (13R)-HPOD from deuterated linoleic acids. (11R)-[2H]Linoleic acid (411 µmol) in buffer A (1.05 ml) was treated with Mn-LO (1.5 µg) at 23 °C. An aliquot was transferred to a cuvette having 1 mm path length, and the absorbance at 235 nm was monitored versus time (○). Aliquots (0.3 ml) were removed at 3.5, 11.5, and 16 min of incubation and subjected to RP-HPLC. Hydroperoxides (3.5-min sample) and linoleic acid were collected, derivatized, and subjected to GC-MS analysis for determination of isotope content. (11S)-[2H]Linoleic acid (321 µmol) was treated with Mn-LO (1.5 µg) in buffer A (1.05 ml) at 23 °C. An aliquot was transferred to a cuvette having 1 mm path length, and the absorbance at 235 nm was monitored versus time (●). Aliquots (0.3 ml) were removed at 4.5, 15, and 49 min of incubation. Products were isolated by RP-HPLC, derivatized, and subjected to GC-MS analysis. Arrow indicates addition of additional Mn-LO (1.5 µg). The estimated rates of formation of (13R)-HPOD in time segments A, B, and C were 7.9, 0.5, and 0.4 nmol min⁻¹ µg⁻¹, respectively.

**DISCUSSION**

Mn-LO purified from the fungus *G. graminis* as described in the accompanying paper (15) catalyzes conversion of linoleic acid into (13R)-HPOD as the major product. This transformation consisted of dioxygenation of a fatty acid possessing a (1Z,4Z)-pentadienyl moiety into a fatty acid hydroperoxide having a 1-hydroperoxy-(2E,4Z)-pentadiene structure, thus satisfying the requirements for classifying Mn-LO as a lipoxygenase enzyme. The aim of the present study was originally to determine the stereochemistry of the biosynthesis of (13R)-HPOD using stereospecifically deuterated linoleic acids. In the course of this work, it became apparent that Mn-LO not only catalyzes transformation of linoleic acid into (13R)-HPOD but also other reactions, which are not characteristic of traditional lipoxygenases.

Experiments with linoleic acids labeled with [2H] in the (11R) and (11S) positions demonstrated that Mn-LO, like soybean lipoxygenase-1, catalyzed abstraction of the pro-S hydrogen from C-11 of linoleic acid (Table II). As was found previously for soybean lipoxygenase (4, 27–31), a pronounced primary isotope effect resulting in accumulation of [2H] in the unconverted substrate was noted in these experiments (Table II, Fig. 6). This result indicated that the first step of the Mn-LO-catalyzed oxygenation, like that of the soybean lipoxygenase-catalyzed oxygenation, consisted of hydrogen abstraction from the bis-allylic methylene group to produce a pentadienyl moiety. The overall steric course of formation of (13R)-HPOD from linoleic acid in the presence of Mn-LO consisted of hydrogen abstraction and oxygen insertion occurring in a suprafacial way (Fig. 7). This was in contrast to the antarafacial stereochemistry repeatedly observed for oxygenations catalyzed by soybean lipoxygenase and other lipoxygenases (4–10).

Analysis of the hydroperoxide product isolated following incubation of linoleic acid with Mn-LO demonstrated the presence of a second, less abundant component in addition to (13R)-HPOD (compound A, Fig. 1). The structure of this compound was determined by ultraviolet spectroscopy and mass spectrometry and by chemical methods. An important clue to the structure was provided by the finding that the hydroperoxy group of compound A was rapidly eliminated upon acid treatment to provide a mixture of 9,10,12- and 9,11,13-octadecadienoic acids (Fig. 2). This type of conversion had earlier been observed with 11-HOD (21, 22), thus indicating that compound A was a bis-allylic hydroperoxide. Reduction of compound A by treatment with sodium borohydride afforded 11-HOD. The geometry of the two double bonds of this product was rigorously established as ZZ by comparison with authentic 11-HOD and with chemically prepared (9E,12Z)-, (9Z,12E)-, and (9E,12E)-11-HOD. Partial hydrogenation of the methyl ester of 11-HOD derived from compound A followed by treatment with (−)-menthoxycarboxyl chloride and oxidative ozonolysis resulted in the formation of chiral fragments whose structures localized the double bonds of compound A to the Δ⁷ and Δ¹⁴ positions and demonstrated that the absolute configuration of C-11 was "S." The experiments thus allowed compound A to be formulated as (11S)-hydroperoxy-(9Z,12Z)-octadecadienoic acid ((11S)-HPOD).
This bis-allylic hydroperoxide was a new oxylipin, although a geometrical isomer of the compound, i.e. methyl (11R,11S)-hydroperoxy-(9Z,12Z)-octadecadienoate, had been prepared by a singlet oxygen reaction (results referred to in Ref. 33). Furthermore, a more unsaturated analog, i.e. 11-hydroperoxyoctadec-12-en-9-ynoic acid, was recently isolated following incubation of the lipoxygenase inhibitor octadec-(12Z)-en-9-ynoic acid with soybean lipoxygenase (34). Biosynthesis of (11S)-HPOD catalyzed by Mn-LO was accompanied by selective loss of the pro-S hydrogen from the carbon dioxygenated (Table II) and thus proceeded with retention of absolute configuration (Fig. 7).

The proportion between (11S)-HPOD and (13R)-HPOD depended on the time of incubation and the substrate concentrations (Fig. 3). Short times of incubation of high substrate concentrations gave the highest yields of (11S)-HPOD and a ratio (11S)-HPOD/(13R)-HPOD equal to 0.31. As shown by RP-HPLC radiocromatographic analysis, the only product present in incubations where (11S)-HPOD had been allowed to disappear was (13R)-HPOD, thus suggesting that (11S)-HPOD was converted into (13R)-HPOD in the presence of Mn-LO. This could be directly demonstrated in experiments where (11S)-HPOD was incubated with Mn-LO and the ultraviolet absorbance due to (13R)-HPOD was monitored versus time. The time course of formation of (13R)-HPOD showed a characteristic sigmoidal shape because of the presence of a lag phase (Fig. 4). The maximum rate of formation of (13R)-HPOD from (11S)-HPOD was ~50% greater than that of formation of (13R)-HPOD from linoleic acid. Conversion of (11S)-HPOD into (13R)-HPOD was suppressed in the presence of high concentrations of linoleic acid (Fig. 3), indicating that (11S)-HPOD and linoleic acid competed for the same catalytic site of Mn-LO. The fact that (11S)-HPOD served as an obligatory intermediate in the formation of (13R)-HPOD. The enzyme glutathione peroxidase, which traps fatty acid hydroperoxides as the corresponding hydroxy compounds, has been successfully utilized to prove the existence of hydroperoxides as intermediates in the biosynthesis of various oxylipins (7, 25, 26). Inclusion of glutathione peroxidase and reduced glutathione in incubations of linoleic acid with Mn-LO resulted in a decreased rate of formation of (13R)-H(PO)D; however, this was not caused by trapping of (11S)-HPOD but by partial inhibition of oxygenation of linoleic acid. Importantly, the ratio of the reduction products, (11S)-HOD/(13R)-HOD, did not increase in the presence of glutathione peroxidase, thus disfavoring the hypothetical sequence linoleic acid → (11S)-HPOD → (13R)-HPOD.

The mechanism of the conversion of (11S)-HPOD into (13R)-HPOD was studied by 18O experiments (Table I). In one set of incubations, linoleic acid was treated with Mn-LO under 18O2 and the hydroperoxides formed were isolated by RP-HPLC. Aliquots were reduced and analyzed by GC-MS as the methyl ester/Me3Si derivatives. As expected, (11S)-HPOD and (13R)-HPOD had both incorporated 18O. Reincubation of [11-18O]-HPOD with Mn-LO resulted in the formation of (13R)-HPOD that retained 39–44% of the 18O label. In a complementary experiment, incubation of (11S)-HPOD under 18O2 led to the formation of (13R)-HPOD that had incorporated 57% 18O. The findings that (13R)-HPOD only partially retained the 18O label when formed from [11-18O2]-HPOD, and that the hydroperoxide had partially incorporated 18O when biosynthesized from (11S)-HPOD under 18O2, showed that the oxygen molecule migrating from C-11 to C-13 during the hydroperoxide rearrangement was subject to exchange with surrounding molecular oxygen. This fact, in turn, necessitated that the conversion of (11S)-HPOD into (13R)-HPOD took place in a stepwise way involving a deoxygenated intermediate. Interestingly, the oxygen exchange also manifested itself in the isotope content of (11S)-HPOD remaining not converted. As seen in Table I, there was a time-dependent loss of 18O from [11-18O2]-HPOD incubated with Mn-LO under ambient air, and an incorporation of 18O from 18O2 into (11S)-HPOD incubated under 18O2. These results gave further support for the existence of a deoxygenated intermediate and additionally in-
dicated that this intermediate could be reversibly oxygenated into (11S)-HPOD. Fig. 7 shows the mechanism proposed for the Mn-LO-catalyzed oxygenation of linoleic acid. The initial step consisted of abstraction of the pro-S hydrogen from C-11 to produce a linoleoyl radical. This intermediate was reversibly converted into (11S)-HPOD via the corresponding (11S)-peroxy radical, or irreversibly converted into (13R)-HPOD via the corresponding (13R)-peroxy radical. Chemical studies on fatty acid autoxidation have demonstrated that conversion of carbon-centered fatty acid radicals into peroxo radicals occurs by reversible binding of dioxygen (35). Furthermore, non-enzymatic free radical rearrangements of fatty acid hydroperoxides have been described (33, 36). In a study of rearrangement of hydroperoxides derived from linoleic acid (36), the methyl ester of (9S)-HPOD (incorrectly referred to as (9R)-HPOD in Ref. 36) was found to yield a mixture of the E,E- and E,Z-isomers of 9- and 13-HPOD methyl esters when kept in hexane solution under O₂. When the reaction was performed under 18O₂, partial incorporation of 18O into the hydroperoxides was observed. Although the 18O experiments of the present study demonstrated the existence of a non-concerted pathway involving a dioxygenated intermediate for the transformation of (11S)-HPOD into (13R)-HPOD, they did not exclude the possibility of an additional mechanism contributing to the formation of (13R)-HPOD, i.e. a direct conversion of the (11S)-peroxy radical into the (13R)-peroxy radical by concerted transfer of the peroxo radical oxygen from C-11 to C-13. Studies of the viability of such a contributing pathway are under way.

It is uncertain whether the mechanism proposed for Mn-LO involving a bis-allylic peroxy radical and a bis-allylic hydroperoxide has any relevance for other lipoxygenases such as soybean lipoxygenase. 11-Hydroperoxyoctadec-12-en-9-ynoic acid has been isolated as one of several products formed from an acetylenic inhibitor, octadec-(12Z)-en-9-ynoic acid, upon incubation with soybean lipoxygenase (34). However, despite extensive studies of soybean lipoxygenase-catalyzed oxygenations, formation of bis-allylic hydroperoxides from polyunsaturated fatty acids has never been reported. In the present study, (11S)-HPOD was tested as a substrate for soybean lipoxygenase with negative result. The possible roles of the corresponding (11S)-peroxy radical, and of (11R)-HPOD or its corresponding peroxy radical, as intermediates in soybean lipoxygenase catalysis remains to be examined.

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