Manganese lipoxygenase of *F. oxysporum* and the structural basis for biosynthesis of distinct 11-hydroperoxy stereoisomers

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Abstract  The biosynthesis of jasmonates in plants is initiated by 13(S)-lipoxygenase (LOX), but details of jasmonate biosynthesis by fungi, including *Fusarium oxysporum*, are unknown. The genome of *F. oxysporum* codes for linoleate 13(S)-LOX (FoxLOX) and for *F. oxysporum* manganese LOX (Fo-MnLOX), an uncharacterized homolog of 13(R)-MnLOX of *Gaumannomyces graminis*. We expressed Fo-MnLOX and compared its properties to Cg-MnLOX from *Colletotrichum gloeosporioides*. Electron paramagnetic resonance and metal analysis showed that Fo-MnLOX contained catalytic Mn. Fo-MnLOX oxidized 18:2 mainly to 11R-hydroperoxyoctadecadienoic acid (HPODE), 13S-HPODE, and 9(S/R)-HPODE, whereas Cg-MnLOX produced 9S, 11S, and 13R-HPODE with high stereoselectivity. The 11-hydroperoxides did not undergo the rapid β-fragmentation earlier observed with 13R-MnLOX. Oxidation of [11S-3H]18:2 by Cg-MnLOX was accompanied by loss of deuterium and a large kinetic isotope effect (>30). The Fo-MnLOX-catalyzed oxidation occurred with retention of the 3H-label. Fo-MnLOX also oxidized 1-lineol-2-hydroxy-glycerol-3-phosphatidylcholine. The predicted active site of all MnLOXs contains Phe except for Ser348 in this position of Fo-MnLOX. The Ser348Phe mutant of Fo-MnLOX oxidized 18:2 to the same major products as Cg-MnLOX. Our results suggest that Fo-MnLOX, with support of Ser348, binds 18:2 so that the proR rather than the pro3 hydrogen at C11 interacts with the metal center, but retains the suprafacial oxygenation mechanism observed in other MnLOXs.—Wennman, A., A. Magnuson, M. Hamberg, and E. H. Oliw. Manganese lipoxygenase of *F. oxysporum* and the structural basis for biosynthesis of distinct 11-hydroperoxy stereoisomers. *J. Lipid Res.* 2015, 56:1606–1615.

Supplementary key words  *Fusarium gloeosporioides* • gene expression • oxygenation mechanism • oxylipins • *Pichia pastoris* • yeast expression • mass spectrometry • *Fusarium oxysporum*

Lipoxygenases (LOXs) belong to a common gene family (1, 2). The catalytic domain is formed by α-helices, which harbor five metal ligands. The 3D structure is known for over 10 enzymes from plants, animals, and bacteria with 13S-LOX of soybean LOX-1 (sLOX-1) and 8R-LOX of *Plexaura homomalla* as old and new prototypes (3–5). The latter was recently crystallized with its substrate in the active site (5). Information on the active site and reaction mechanism has also been gained by site-directed mutagenesis and by analysis of various substrates, deuterated fatty acids, and LOX inhibitors (1, 6). With few exceptions, LOXs oxidize polyunsaturated fatty acids with one or more cis,cis-1,4-pentadiene units to hydroperoxides, which in many cases are precursors of biological mediators (1, 6, 7). Mammalian LOXs initiate biosynthesis of leukotrienes and epoxy alcohols, which are involved in allergic inflammation, asthma, formation of the skin-water barrier, and cancer development (6, 8). Plant LOXs form jasmonates and related oxylipins with functions in defense against pathogens and in wound healing (9, 10).

The N-terminal parts of mammalian and plant LOXs usually consist of ß-sheets with homology to polycystin-1/LOX/α-toxin (PLAT) domains with regulatory functions. The N-terminal domains of fungal LOXs lack this homology, but they often contain a secretion signal (11, 12).

Abbreviations: AfMnLOX, *Aspergillus fumigatus* manganese lipoxygenase; Cg-MnLOX, *Colletotrichum gloeosporioides* manganese lipoxygenase; CP, chiral phase; EPR, electron paramagnetic resonance; FeLOX, iron lipoxygenase; Fo-MnLOX, *Fusarium oxysporum* manganese lipoxygenase; HPODE, hydroperoxyoctadecadienoic acid; 9S(9H)P(O)DE, 9-hydroxy(pero)xy-10,12-octadecadienoic acid; 11S(9H)P(O)DE, 11-hydroxy(pero)xy-9Z,12Z-octadecadienoic acid; 13S(9H)P(O)DE, 13-hydroxy(pero)xy-9Z,11E-octadecadienoic acid; HPOPTE, hydroperoxyoctadecatrienoic acid; 9S(9H)P(O)TE, 9-hydroxy(pero)xy-10,12,15-octadecatrienoic acid; 11R-H(9H)P(O)TE, 11-hydroxy(pero)xy-9Z,12Z,15-octadecatrienoic acid; 13R-H(9H)P(O)TE, 13-hydroxy(pero)xy-9Z,11E,15Z-octadecatrienoic acid; ICP-AES, inductively coupled plasma atomic emission spectroscopy; LOX, lipoxygenase; MnLOX, manganese lipoxygenase; Mo-MnLOX, *Mangifera indica* manganese lipoxygenase; RP, reversed phase; sLOX-1, soybean lipoxygenase-1; TIP, triphenylphosphine.

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Fungal pathogens may secrete LOXs during invasive growth to generate reactive oxygen species and lysis of membranes. Fungal LOXs may also regulate biosynthesis of secondary metabolites and toxins, including biosynthesis of jasmonates (11, 13–17).

Fusarium oxysporum was found to produce jasmonic acid over 20 years ago, but the mechanism is still unknown (15). This has sparked interest in the pattern of oxygenation of C₁₈ fatty acids by this fungus. The genome codes for several dioxygenases of linoleic and α-linolenic acid: 9dioxygenase-allene oxide synthase, 10Rdioxygenase-epoxy alcohol synthase, a related 9Rdioxygenase, and two LOXs designated FoxLOX and F. oxysporum manganese LOX (Fo-MnLOX) (18, 19) (Fig. 1).

![Fig. 1. Overview of oxylipins formed by F. oxysporum and a phylogenetic tree of two LOX prototypes from soybean and coral and four fungal Mn- and FeLOXs. A: Overview of oxylipin biosynthesis by F. oxysporum. 9S-dioxygenase-allene oxide synthase (9S-DOX-AOS; FOXB_01332), 9Rdioxygenase (9R-DOX; FOXB_09952) (confirmed by recombinant expression; L. Sooman and E. H. Oliw, unpublished observations), and 10Rdioxygenase-epoxy alcohol synthase (10R-DOX-EAS; FOXB_03425) of distinct subfamilies with homology in their dioxygenase domains. The putative Fo-MnLOX has not been characterized, whereas FoxLOX is an iron 13S-LOX (11). B: Phylogenetic tree of informative sequences with homology to FoxLOX and Fo-MnLOX. Soybean 13S-LOX is often designated sLOX-1. The GenBank numbers are from top to bottom: FoxLOX (EXK38530), Pleurotus ostreatus (BA199788), P. homomalla (ACC47283), Glycin max (P08170), F. oxysporum (EGU80482; FOXB_09904), C. gloeosporioides (EQB45907; CGLO_15145), and G. graminis (AAK81882). The GenBank accession number of an identical protein to FoxLOX is EXK38530; the accession locus of FoxLOX is FOXG_04807 at the Broad Institute.](image-url)

Plants form jasmonates from the 13S-hydroperoxide of 18:3n-3, which is sequentially transformed by plant allene oxide synthase (CYP74) and by allene oxide cyclase to 12-oxophytodienoic acid, the precursor of jasmonates (20). Fo-MnLOX shows 13S-LOX activities and could provide the hydroperoxide precursor for jasmonate biosynthesis (11). Fo-MnLOX might also contribute, but little is known about this enzyme other than its sequence homology to the MnLOX subfamily (11).

The prototype of the MnLOX family is 13R-MnLOX of the take-all fungus of wheat, Gaumsannomyces graminis (21–24). Other members are 9S-MnLOX of the rice stem root fungus, Magnaporthe salvinii (25), Mo-MnLOX of the rice blast fungus, Magnaporothe oryzae (A. Wennman et al., unpublished observation), and 13-MnLOX of Aspergillus fumigatus and homologs within Fusarium, Colletotrichum, Aspergillus, and Penicillium (11, 12, 22, 23) (Fig. 1B).

Fungal LOXs with catalytic Fe, e.g., FoxLOX of F. oxysporum and the recently characterized 13-LOX of the oyster mushroom, Pleurotus ostreatus (26), form a separate subfamily with larger sequence similarities to plant and animal LOXs than to MnLOXs (Fig. 1B) (11, 12).

13R-MnLOX was recently crystallized (27), but the 3D structure has not yet been resolved. Electron paramagnetic resonance (EPR) analysis suggests that Fe and Mn are ligated in Fe- and MnLOXs in a similar way in analogy with other Fe/Mn enzymes (21, 28). The two ion pairs, Fe²⁺/Fe³⁺ and Mn²⁺/Mn³⁺, differ in redox potentials, 0.77 and 1.5 V, respectively (29, 30). This difference is likely reduced by the Mn coordinating amino acid residues to allow oxidation and recycling of the metal center (29).

F. oxysporum and Colletotrichum gloeosporioides belong to the top ten fungal pathogens of molecular biology, and they can devastate the harvest of a wide range of fruits and vegetables (31, 32). Their MnLOXs are likely secreted and might contribute to the pathogenic process by oxidation of plant lipids and biosynthesis of oxylipins, and they are structurally related (Fig. 1B). For these purposes we chose to express and characterize these enzymes, which could be more suitable than 13R-MnLOX for crystallization and diffraction.

MATERIALS AND METHODS

Materials

HPLC solvents (LiChrosolv) and routine chemicals were from Merck or Sigma-Aldrich. The18:2n-6 (99%), yeast nitrogen base, and α-mannosidase were from Sigma-Aldrich. Endoglucosidase H was obtained locally. The 18:3n-3 (99%), 20:2n-6 (99%), 1,2-dilinoleoylglycero-3-phosphatidylcholine, and L-sphingosylcer-3-phosphatidylcholine were from Larodan. [11H]18:2n-6 (>99% ²H) was prepared as described (33). Fatty acids were dissolved in ethanol and stored in stock solutions (50–100 mM) at –20°C. 13Hydroperoxycytadecadienoic acid (HPODE), 13R-HPODE, 13R-hydroperoxycytadecatrienoic acid (HPOTR-E), 9S-HPODE, and 9S-HPOTR-E were obtained by biosynthesis (sLOX-1, 13R-MnLOX, tomato LOX) and purified by reversed phase (RP)-HPLC or by open-column silic acid chromatography (SepPak/SiO₂). Racemic 9- and 13-HPODE were obtained by photo
amplified in the ered glycerol yeast medium in baffled flasks at 28°C (200 rpm; 48 h).

which predicts Cg-MnLOX with a duplicate error (see text for 17993 (EWZ28287).

are: FOPG_18781 (EXL64974), FOQG_18345 (EXK76927), and FOZG_1608 Journal of Lipid Research

and His 126  (in IQIHGG) and the codons of Asp 124  and His 125  (in MnLOX expression constructs

ris Pichia pastoris

MnLOX sequences aligned and ended PheTyrLeuSerVal.

was deleted from the expression construct so that the Fo- and Cg-(Cg-MnLOX) was duplicated (34 amino acids); this extension

dicted with the aid of the SignalP 4.1 server. The C-terminal end

sion was induced by addition of 0.5% methanol daily for 3–5

then harvested. The yeast cells were precipitated by centrifugation.

sion cassettes were from Millipore (Amicon Ultra-15).

racel OB-H (250 × 4.6 mm) was purchased from Daicel, and silicic

250 × 2 mm) and ReproSil Chiral AM (5

PCR were obtained from TIB Molbiol (Berlin, Germany). Chemi-

on an ÄKTA FPLC (Pharmacia) or a peristaltic pump (P-3; Pharm-

The proposed open reading frames of the MnLOXs from

2  The GenBank accession number is EGU80482.1 (FOXB_09004),

F. oxysporum’’ and C. gloeosporioides’’ were based on one intron (64 nt and 59 nt, respectively), predicted between the codons of Ile25 and His126 (in IQQHGG) and the codons of Asp124 and His125 (in IQDHGG), respectively. Their secretion signals of 16 nt were predicted with the aid of the SignalP 4.1 server. The C-terminal end

Site-directed mutagenesis of MnLOX

pPICZaa, FoMnLOX was modified by replacements of Ser148 with a Phe residue and Leu256 with an Arg residue, with the aid of site-directed mutagenesis using Pfu polymerase. The PCR products were restricted with DpnI, analyzed by agarose gel electrophoresis, and used to transform E. coli (NEB5a), purified by Nucleasebond AX (Xtra Midi kit), and linearized with Pmel and SacI, respectively. P. pasto-

Site-directed mutagenesis of FoMnLOX

Electrode potentials were measured on a dual beam spectrophotometer (Shimadzu UV-2101PC). The enzyme was mixed with 100 μM 18:2n-6 or 18:3n-3 in 0.1 M NaBO

The enzyme activity sometimes declined at the third day.

Protein expression and purification

Phleomycin-resistant P. pastoris colonies were grown in buff-

2 The GenBank accession number is EQB45907 (CGLO_15145), which predicts CgMnLOX with a duplicate error (see text for details).

Solid (NH4)2SO4 (to 25% saturation) was added to the superna-

tant and pH was adjusted to 6.5 with 10 M KOH. The proteins, which were precipitated by (NH4)2SO4, were removed by centrifuga-

tion (18,000 g 20 min, 4°C), and the supernatant, which contained most of the LOX activity, was stored at −80°C until analysis.

After thawing, the supernatant (200–500 ml) was filtered and loaded (3 ml/min) on a Butyl-Sepharose column (25–32 ml, 21°C) equilibrated with 25 mM KHPO4 (pH 6.5) and 1 M (NH4)2SO4 on an ÄKTA FPLC (Pharmacia) or a peristaltic pump (P-3; Phar-

After thawing, the supernatant (200–500 ml) was filtered and loaded (3 ml/min) on a Butyl-Sepharose column (25–32 ml, 21°C) equilibrated with 25 mM KHPO4 (pH 6.5) and 1 M (NH4)2SO4 on an ÄKTA FPLC (Pharmacia) or a peristaltic pump (P-3; Pharm-


duced with 25 mM KHPO4 (pH 6.5), and the fractions were assayed for LOX activity by UV spectroscopy (25). The active frac-

tions were pooled, the volume was reduced by diafiltration (Ul-

Enzyme assay.

Fatty acids were incubated with the Fo- and Cg-

MnLOX in 0.1 M NaBO4 buffer (pH 9.0). The products were identified as described below. LOX activity was measured on a
dual beam spectrophotometer (Shimadzu UV-2101PC). The enzyme was mixed with 100 μM 18:2n-6 or 18:3n-3 in 0.1 M NaBO4 (pH 9.0), and the UV absorbance was followed at 235 and 237 nm, respectively. Kinetic isotope deuterium effects were determined by incubation of the enzymes with 100 μM [115H]18:2n-6 (>99% H) and 100 μM 18:2n-6, and by comparing the increase in UV absorbance at 235 nm. The reaction rate was estimated from the linear part of the curve.

The apparent Km of Fo-MnLOX was first estimated by plotting the reaction rate with 18:2n-6 (5–100 μM) from the linear rate of biosynthesis of cis-trans conjugated 9- and 13-HPODE (UV analysis). We next examined the biosynthesis of 11-HPODE, which was estimated from the amounts of 9- and 13-HPODE by RP-HPLC of samples obtained during the linear rate and zoom scan MS analysis with integration of the signal intensities of the carboxylate anions of 11-HPODE and 9- and 13-HPODE (m/z 311). These ratios, 11-HPODE/9-HPODE+13-HPODE), appeared to decrease with increasing linoleate concentrations. The apparent Kcat was calculated with the aid of the enzyme for 11-HPODE biosynthesis.

Site-directed mutagenesis of Fo-MnLOX.

We investigated two positions of Fo-MnLOX by replacement, Ser348Phe and Leu530Arg and Fo-MnLOX·Ser348Phe and Fo-MnLOX·Leu530Arg and were expressed and partially purified by hydrophobic interaction chromatography as above.

LC-MS/MS analysis

Products were extracted by isolation on C18 silica cartridge (SepPak/C18) and eluted with ethyl acetate (4 ml). RP-HPLC-

MS/MS analysis was performed with a Surveyor MS pump (Thermo-Fisher), on an octadecyl silica column (5 μm; 2 × 150 mm; Phenomenex, equipped with a 4 × 2 mm guard cartridge, Phenomenex), which were eluted at 0.3 ml/min with methanol/water/acetic acid (750/250/0.05) for monohydro(pero)xy fatty acids. The effluent was subjected to electrospray ionization in a linear ion trap mass spectrometer (LTQ, Thermo-Fisher). The heated transfer capillary was set at 315°C, the ion isolation width
usually at 1.5 amu (5 amu for hydroperoxides and for $^3$H-labeled metabolites), and the collision energy at 35 (arbitrary scale). PGE$_{650}$ was infused for automatic tuning, which adjusted the tube lens near 100 V.

Normal phase HPLC-MS/MS was performed with a silica column (5 μm, 250 × 2 mm; Kromasil 100 A), usually eluted with hexane/isopropanol/acetacid, 97/3/0.05, at 0.5 ml/min. For chiral phase (CP) separation of hydroperoxy fatty acids, we used a Reprosil Chiral-NR column eluted with hexane/isopropanol/acetacid, 99/1/2/0.05, at 0.6 ml/min (34). Hydroxy fatty acids were analyzed on Chiralcel OB-H (eluted at 0.5 ml/min with hexane/isopropanol/acetacid, 95/5/0.05) or on Reprosil Chiral AM (eluted at 0.1–0.2 ml/min with hexane/ethanol/acetacid, 95/5/0.05). The eluents of these columns were combined inline with isopropanol/water (3/2; 0.25 and 0.1 ml/min, respectively) and introduced by electrospray ionization into the linear ion trap mass spectrometer (LTQ).

**Metal analysis**

EPR measurements were performed on a Bruker ELEXYS E500 spectrometer using an ER040X SuperX microwave bridge, in a Bruker SHQ601 cavity equipped with an Oxford Instruments continuous flow cryostat. The measurement temperature was 7 K, using an ITC503 temperature controller (Oxford Instruments) and liquid helium as coolant. Signal processing was performed using the Xepr software package (Bruker). EPR analysis of Fo-MnLOX [0.15 mM in 0.2 ml 25 mM HEPES (pH 7.0)/100 mM NaCl/10% glycerol] was performed before and after denaturation of the protein with H$_2$SO$_4$.

Metal content in the protein sample was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Spectroflame P). The protein sample was diluted in buffer with 5 mM EDTA and then washed three times by diafiltration with Spectroflame P. The protein sample was diluted in buffer with 5 ml with above-mentioned low metal buffer and the protein concentration was estimated to be 0.49 mg/ml by UV absorbance as described above. The samples were prepared by mixing 5 μl of the precipitated sample with 125 μl of 100 mM H$_2$SO$_4$.

The Mn and Cu effect on catalytic activity was analyzed by pre-incubation of the purified enzyme with 1 μM and 10 μM CuSO$_4$ and with 1μM and 10 μM MnCl$_2$. The reaction rate was followed with UV absorbance as described above. The samples were prepared by mixing 5 μl of the precipitated sample with 125 μl of 100 μM 18:2n-6 in borate buffer.

**Bioinformatics.** ClustalW (DNASTAR) was used for sequence alignments. SWISS-MODEL was used for modeling of Fo-MnLOX with 8RLOX as a template (37). PyMOL Molecular Graphics system version 1.7.4 (Schrödinger, LLC) was used for visualizing the model.

**RESULTS**

**Expression and purification of Fo- and Cg-MnLOXs**

Fo- and Cg-MnLOXs were expressed in baffled flasks and secreted to the growth medium. Attempts to precipitate the enzymes with (NH$_4$)$_2$SO$_4$ at 60% saturation were unsuccessful. The enzymes could be purified by hydrophobic interaction chromatography (Butyl-Sepharose 4F) as described in the Materials and Methods. This resulted in capture of 98% of the applied enzyme activity and an 18-fold increase in specific activity from 1.3 to 23.7 nmol·mg$^{-1}$·min$^{-1}$. Concentration by diafiltration and gel filtration resulted in 74-fold purification and a specific activity of 96 nmol·mg$^{-1}$·min$^{-1}$.

SDS-PAGE analysis of Fo-MnLOX during the purification steps and after deglycosylation is shown in Fig. 2. The theoretical molecular mass of Fo-MnLOX was 66.7 kDa and Cg-MnLOX was slightly smaller, 66.2 kDa. The predicted isoelectric points were also similar, 6.9 and 6.4, respectively. Secreted proteins by *P. pastoris* can be subject to O- and N-linked glycosylation, mainly with long chains of mannose residues (38). SDS-PAGE analysis showed a broad band between 80 and 90 kDa that could be reduced to about 65 kDa by deglycosylation with α-mannosidase and endoglycosidase H.

**Catalytic properties of Fo- and Cg-MnLOXs**

Fo- and Cg-MnLOXs oxidized 18:2n-6 to 11-HPODE, 9-HPODE, and 13-HPODE (Fig. 3A). The 18:3n-3 was oxidized by Fo- and Cg-MnLOXs to 11-HPOTrE as the main product along with small amounts 13- and 9-HPOTrE (Fig. 3B). Fo-MnLOX also formed small amounts of 10-HPOTrE. 11-HPOTrE also accumulated along with 9- and 13-HPOTrE after prolonged incubation times (after the end of the linear increase in UV absorbance).

Steric analysis showed that Cg-MnLOX oxidized 18:2n-6 to 9S- and 13R-HPODE almost exclusively (>95% S and R, respectively), whereas Fo-MnLOX formed 13S- and 13R-HPODE in a 9/1 ratio and rac-9-HPOTrE (Fig. 3C, D). 13S-HPODE was obtained with less stereoselectivity in some experiments, possibly due to nonenzymatic decomposition of 11-HPOTrE.

Incubation of 11-HPOTrE synthesized by Fo-MnLOX with the previously characterized 13R-MnLOX revealed almost exclusive transformation to 9R-HPODE (Fig. 4A). 13R-MnLOX transforms 11S-HPODE to 13R-HPODE and 11R-HPODE to 9R-HPODE (30). We therefore conclude...
that Fo-MnLOX forms the R enantiomer of 11-HPODE. This is in contrast to previously characterized MnLOXs that mainly formed the S enantiomer.

We confirmed that Cg-MnLOX formed 11-HPOTrE, which was transformed by 13R-MnLOX to 13-HPOTrE (and not to 9-HPOTrE). It was thus identified as 11R-HPOTrE, which is the same 11-HPOTrE stereoisomer formed by 13R-MnLOX and transformed to 13-HPOTrE. It is noteworthy that the R or S configuration at C-11 of 18:2(n-6) and 18:3(n-3), by Cahn-Ingold-Prelog nomenclature rules, is influenced by the 15Z double bond, which results in different R and S chirality at the 11 position of HPOTrE and HPODE, respectively, even though the hydrogen is facing in the same side.

CP-HPLC-MS/MS analysis and partial separation of a mix of 11S- and 11R-HODE, which are formed by Cg- and Fo-LOX, respectively, is shown in Fig. 4B.

Incubations of [11S-2H]18:2(n-6) (100 μM) revealed interesting differences between Cg-MnLOX and Fo-MnLOX. The reaction catalyzed by the former enzyme involved loss of the deuterium label accompanied by a strong (>30) kinetic isotope effect (data not shown), whereas the Fo-MnLOX-catalyzed reaction proceeded with retention of the...
label and a small (~2) isotope effect (Fig. 4C). This low number is characteristic of secondary deuterium kinetic isotope effects (39). The MS analysis of the anions of the hydroperoxides formed by Fo-MnLOX (Fig. 4C, insert) details the retention of the deuterium label, which was further supported by the MS/MS spectrum of 11-HODE (Fig. 4D). The latter shows retention of the label, except for two odd numbered ions [m/z 169 and 151 (169-18)]. We therefore conclude that 11R-HPODE, produced by Fo-MnLOX, is formed by suprafacial hydrogen abstraction and oxygen insertion. Other MnLOXs synthesize 11S-HPODE by suprafacial hydrogen abstraction and oxygen insertion (24, 25).

Analysis of the rate of products formed from 18:2n-6 by UV analysis (235 nm) at different substrate concentrations is shown in Fig. 4E. These data indicated an apparent UV analysis (235 nm) at different substrate concentrations purported by the MS/MS spectrum of 11-HODE (Fig. 4D). The latter shows retention of the deuterium label, which was further supported by the MS/MS spectrum of 11-HODE (Fig. 4D). The latter shows retention of the label, except for two odd numbered ions [m/z 169 and 151 (169-18)]. We therefore conclude that 11R-HPODE, produced by Fo-MnLOX, is formed by suprafacial hydrogen abstraction and oxygen insertion. Other MnLOXs synthesize 11S-HPODE by suprafacial hydrogen abstraction and oxygen insertion (24, 25).

Oxidation of other fatty acids by Fo-MnLOX

We screened fatty acids (100 μM) for apparent oxygenation by purified Fo-MnLOX (0.6 μM) by UV analysis (235 or 237 nm). Under these conditions, 18:2n-6 was oxidized with kcat 0.73 ± 0.07/min (n = 3). The rates of oxidation of 20:2n-6 and 20:3n-3 were almost identical, kcat 0.64 ± 0.15/min and 0.67 ± 0.7/min, respectively. LC-MS analysis showed that Fo-MnLOX oxidized 20:2n-6 efficiently at both C15 and C11. The oxidation of 20:2n-6 and 20:3n-3 implies that the carbon chain elongation was not critical for positioning of the 1,4-pentadiene in the active site of Fo-MnLOX. Shortening of the carbon chain or multiple double bonds affected catalysis. No significant oxidation of 16:3n-3, 18:4n-3, and 20:4n-6 could be detected.

The 18:3n-3, which forms 11-HPOTrE as a main metabolite (Fig. 3B), was oxidized to 9- and 13-HPOTrE at a much lower rate (0.09 ± 0.02/min) compared with 18:2n-6, 20:2n-6, and 20:3n-3.

Both Fo-MnLOX and Cg-MnLOX oxidized soybean Lα-lipoxyglycerol-3-phosphatidylcholine as judged from a rapid and steady increase in UV absorbance at 235 nm, as shown for Fo-MnLOX in comparison with 18:2n-6 (Fig. 4E). Lα-lipoxyglycerol-3-phosphatidylcholine contained ~47% 18:2, ~2-3% 18:3, and ~50% of other fatty acids (mainly 16:0, 18:0, and 18:1) as judged from alkaline hydrolysis, extractive isolation, and LC-MS analysis of carboxylate anions. We therefore compared the rate of oxidation of 200 μM Lα-lipoxyglycerol-3-phosphatidylcholine with that of 100 μM 18:2n-6. The former was oxidized at a slightly lower rate, 0.66 ± 0.07/min, than 18:2n-6 (see above). We conclude that 1-linoleyl-2-hydroxyglycerol-3-phosphatidylcholine and 18:2n-6 are oxidized at almost similar rates. 1,2-Dilinoleylglycerol-3-phosphatidylcholine was a poor substrate (Fig. 4F).

These results, taken together, suggest that fatty acids likely bind tail first to the active site of Fo- and Cg-MnLOX.

Metal analysis

Mn, Fe, and Cu contents of purified recombinant Fo-MnLOX enzyme (0.49 mg/ml) were determined by ICP-AES to be 254.4, 4.6, and 154.6 ng/ml, respectively (Fig. 5B). The Mn/protein ratio was 0.6/1. We can therefore deduce that the catalytic metal of Fo-MnLOX is Mn and not Fe. The Cu content in the protein sample compared with the blank was high, suggesting binding of Cu to the enzyme in a ratio of 0.3/1.

EPR analysis of Fo-MnLOX (~0.15 mM in 0.2 ml 25 mM HEPEs (pH 7.0)/100 mM NaCl/10% glycerol) before denaturation showed a strong Cu2+ signal, but denaturation with H2SO4 led to the appearance of a prominent and characteristic sextet of Mn2+ (between 3,100 and 3,700 G) (Fig. 5). This is in analogy with the release of protein-bound Mn2+ by denaturation of 13RMnLOX [compare (22)]. From these results, we conclude that the catalytic metal of Fo-MnLOX is likely Mn. The detection of Cu2+ was unexpected, as the sample was prepared to reduce contamination by divalent ions by diafiltration as described in the Materials and Methods.

Preincubation of the purified enzyme with 1 or 10 μM MnCl2 did not increase the catalytic activity. Preincubation with 1 μM or 10 μM CuSO4 abolished the oxidation of 18:2n-6. It is possible that Mn2+ and Cu2+ might reduce the hydroperoxides in the sample and thereby prevent the catalytic metal from being oxidized to its active form.

Replacements of Fo-MnLOX and a 3D model based on 8R-LOX

Fo-MnLOX-Leu530Arg had little influence on the product profile formed from 18:2n-6 in comparison to native Fo-MnLOX.
recombinant Fo-MnLOX. Fo-MnLOX·Ser348Phe altered the product profile (Fig. 6A). 13R and 13S-HODE were formed in a ratio of 74/26, and 9R and 9S-HODE in a ratio of 6/94. 11-HODE consisted mainly of the 11S stereoisomer (Fig. 6B). The relative amounts of 9- and 13-HPODE were unchanged. The rate (UV analysis) of oxidation of 18:2n-6 by this mutant after capture on the Butyl-Sepharose column was 0.025 AU/min with 0.5 mg/ml protein.

Fo-MnLOX·Ser348Phe oxidized both 18:2n-6 and 1-linoleoyl-2-hydroxy-glycero-3-phosphatidylcholine of L-lysoglycero-3-phosphocholine, as judged from UV analysis, and the rate of oxidation of the latter appeared to be reduced by ~15%. This suggests that substrates enter the active site of the mutant with the ω end (“tail”) first in, the same as it was in the native Fo-MnLOX.

We prepared a model of Fo-MnLOX with 8R-LOX (pdb: 3fg1) as template (Fig. 7). The figure illustrates the position of Ser348 (Fig. 7A) and the Phe residue after replacement (Fig. 7B), as well as Leu337 and Phe338, previously shown to be important for binding of the substrate in the active site. Leu330 is not shown.

DISCUSSION

We report as our main finding that the MnLOX homolog of F. oxysporum, Fo-MnLOX, retained the same oxidation mechanism as 9S- and 13R-MnLOX, but formed the unexpected 11R stereoisomer of 11-HPODE as a major metabolite (Fig. 8A). In contrast, the Cg-MnLOX homolog formed the expected 11S stereoisomer of 11-HPODE in analogy with 13R-MnLOX.

Fo-MnLOX forms 11R-HPODE by suprafacial hydrogen abstraction and oxygen insertion, whereas Cg-, 9S-, and 13R-MnLOX form 11S-HPODE by the same mechanism. Fo-MnLOX apparently binds 18:2n-6 so that the proR hydrogen at C-11 is presented to the metal center, whereas the proS hydrogen is presented in Cg-, 9S-, and 13R-MnLOXs. 11R-HPODE is also formed by the mini iron LOX (FeLOX) of the cyanobacterium Cyanotheca sp., but this occurs by antarafacial hydrogen abstraction and oxygenation (40). One unique property of MnLOXs in comparison with FeLOXs appears to be the suprafacial hydrogen abstraction and oxygenation mechanism (24, 25).

Fig. 6. CP-HPLC-MS analysis of oxidation products of 18:2n-6 formed by Fo-MnLOX·Ser348Phe. The products were reduced to alcohols with TPP and separated on Reprosil Chiral AM. A: Separation of stereoisomers of 13- and 9-HODE (flow 0.15 ml/min) in the same chromatogram. B: Separation of stereoisomers of 11-HODE (flow 0.1 ml/min). The top chromatogram represents analysis of 11-HODE formed by Fo-MnLOX·Ser348Phe. 11-HODE eluted in a single peak. The bottom chromatogram shows separation after addition of a small amount of 11R-HODE to the top sample and partial separation of stereoisomers.

Fig. 7. A hypothetical model to illustrate the differences between Fo-MnLOX and Fo-MnLOX·Ser348Phe in the active site. A: Fo-MnLOX. B: Fo-MnLOX·Ser348Phe. The pentadiene unit (C-9 to C-13) of 18:2n-6 is positioned in the active sites for abstraction of the proR hydrogen in (A) and the proS hydrogen in (B).

11-Hydroperoxides are not subjected to β-fragmentation by Fe- and Cg-MnLOXs or by FeLOXs at significant rates in comparison with 13R-, 9S-, and Mo-MnLOX (30, 41) (A. Wennman et al., unpublished observation). It is doubtful whether the reduction potential of the protein-bound Fe2+/Fe3+ is sufficient for β-fragmentation (40). A catalytic redox center with Mn2+/Mn3+ might be required, but Fo- and Cg-MnLOX illustrate that steric factors also might be important.

The sequences of Fo- and Cg-MnLOXs can be aligned with 65% amino acid identities, yet Fo- and Cg-MnLOXs apparently oxidize 18:2n-6 to hydroperoxides with different chirality. Fo- and Cg-MnLOXs also oxidize L-lysophosphatidylcholine in analogy with 13R-MnLOX (41). This observation could be in agreement with a common substrate channel, which binds the omega end of fatty acids first and with the carboxylate group (head) at the orifice.

The 3D structure of 8R-LOX with bound substrate, arachidonic acid, was recently published (5). The active site is U-shaped and mainly surrounded by hydrophobic amino acid residues. Table 1 illustrates the sequence homology...
within the tentative active sites of MnLOXs, and for comparison, the active site of 8R-LOX (5, 42). The most conspicuous differences between four MnLOXs and Fo-MnLOX are replacement in Fo-MnLOX with Ser348 and Leu530 instead of Phe and Arg in the corresponding position in the other four MnLOXs. Table 1 also illustrates that the geometry of the Mn metal ligands may not be uniform within the MnLOX subfamily, as judged from their primary sequences: The first described enzymes, 13×S-LOX and 13SHPODE are formed by suprafacial hydrogen abstraction and oxygen insertion. B: Hypothetical mechanism for Fo-MnLOX·Ser348Phe with abstraction of the proS hydrogen at C-11. The catalytic metal is marked by an orange sphere. The opposite head-to-tail substrate orientation in (B) compared with (A) seems unlikely, as both enzymes oxidized 1-linoleoyl-2-hydroxy-glycero-3-phosphatidylcholine.

A remarkable and unexpected feature of Fo-MnLOX·Ser348Phe was the marked increase in chirality of 9-HPODE, from racemic to 94% S configuration. Enzymes are believed to be optimized for their catalysis, and replacements in their active sites are not, to the best of our knowledge, known to improve stereoselectivity in this way. It may therefore be argued that the genomic sequence of Fo-MnLOX could be incorrect at the Ser348 position. This appears to be unlikely. It was reassuring to find that there are hypothetical proteins from three F. oxysporum formae speciales, and these proteins are identical to Fo-MnLOX.

EPR and metal analysis confirmed that Mn was present in large excess over Fe. EPR analysis before and after denaturation showed that Mn was mainly protein bound. EPR and metal analysis also revealed that Cu was present at high concentration in spite of purification, washing with EDTA buffer followed by diafiltration. The EPR signal of Cu2+ was not augmented by protein denaturation to the same extent as the EPR signal of Mn2+. According to the Irving-Williams series of metal binding to proteins, Cu binds more strongly than both Fe and Mn (47). The high copper content in the protein sample could be due to either nonspecifically protein-bound Cu2+ or specifically bound Cu2+ at the active site. In the latter case, a catalytic role of Cu2+/Cu2+ in lipoxygenation is unprecedented and therefore seems unlikely.

It is of interest to compare Fo-MnLOX and FoxLOX of F. oxysporum. Both enzymes oxygenate 18:2n-6, but FoxLOX forms 13SHPODE almost exclusively (11). Fo-MnLOX oxidized all three possible positions of 18:2n-6, and 13S and 11R-HPODE were formed with stereoselectivity. The oxidation of 18:3n-3 by Fo-MnLOXs led to 11S-HPOTrE as the main metabolite (11S or 11R is defined by the Cahn-Ingold-Prelog nomenclature rules, which takes the presence or absence of the 15Z double bond into account). 11S-HPOTrE cannot be transformed to jasmonates, as far as is known. FoxLOX therefore remains the prime candidate LOX for fungal biosynthesis of jasmonates from 13SHPOTrE in the plant pathway.
Fo-MnLOX, FoxLOX, and the three dioxygenases in the lipidome of *F. oxysporum* illustrate that we lack information on the function and biological activities of a series of oxylipins (Figs. 1A, 4). The secretion signals of both fungal Fe- and Mn-LOXs suggest an extracellular function in plant-pathogen interactions. Many MnLOXs are expressed by fungal pathogens, which grow within the plant tissue in analogy with *G. graminis*, *M. salvinii*, and *M. oryzae*. Human LOXs contribute to lysis of cell membranes, e.g., in reticulocytes and mitochondria (1). Fungal LOXs could have a similar action on plant cellular membranes during invasive growth.

### CONCLUSION

Fo- and Cg-MnLOXs differ from 13R-MnLOX in several respects. First, Fo-MnLOX forms 11R-HPODE by suprafacial hydrogen abstraction and oxygenation. Second, 11-hydroperoxides of 18:2n-6 and 18:3n-3 were subject to rapid β-fragmentation by 13R-MnLOX, but not by Fo- and Cg-MnLOX. Third, the four residues between two of the ten tentative histidine Mn ligands of Fo- and Cg-MnLOXs are also found in FeLOXs and Mo- and Af-MnLOXs, but are replaced by three residues in 13R- and 9S-MnLOXs. The unique reaction mechanism of Fo-MnLOX can be explained by substitution of Phe with Ser during evolution.

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### REFERENCES

1. Kuhn, H., S. Banthiya, and K. van Leyen. 2015. Mammalian lipoxygenases and their biological relevance. *Biochim. Biophys. Acta*. 1851: 308–330.

2. Horn, T., S. Adel, R. Schumann, S. Sur, K. R. Kakularam, A. Polamarasetty, P. Redanna, H. Kuhn, and D. Heydeck. 2015. Evolutionary aspects of lipoygenases and genetic diversity of human leukotriene signaling. *Prog. Lipid Res.* 57: 13–39.

3. Minor, W., J. Steczko, B. Stec, Z. Otwinowski, J. T. Bolin, R. Walter, and B. Axelrod. 1996. Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. *Biochemistry*. 35: 10687–10701.

4. Neau, D. B., N. C. Gilbert, S. G. Bartlett, W. Beoglin, A. R. Brash, and M. E. Newcomer. 2009. The 1.85 Å structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. *Biochemistry*. 48: 7906–7915.

5. Neau, D. B., G. Bender, W. E. Beoglin, S. G. Bartlett, A. R. Brash, and M. E. Newcomer. 2014. Crystal structure of a lipoxygenase in complex with substrate: the arachidonic acid-binding site of 8R-lipoxygenase. *J. Biol. Chem.* 289: 31905–31913.

6. Høegh-Jensen, J. Z., and C. D. Funk. 2011. Lipoygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem. Rev.* 111: 5866–5898.

7. Andreou, A., F. Brodhun, and I. Feussner. 2009. Biosynthesis of oxylipins in non-mammals. *Prog. Lipid Res.* 48: 148–170.

8. Zheng, Y., H. Yin, W. E. Beoglin, P. M. Elias, D. Crumrine, D. R. Beier, and A. R. Brash. 2011. Lipoxygenases mediate the effect of essential fatty acid in skin barrier formation: a proposed role in releasing omega-hydroxyceramide for construction of the corneocyte lipid envelope. *J. Biol. Chem.* 286: 24046–24056.

9. Wasternack, C., and E. Kombrink. 2010. Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. *ACS Chem. Biol.* 5: 65–77.

10. Vicente, J., T. Cascon, B. Vicedo, P. Garcia-Agustin, M. Hamberg, and C. Castresana. 2012. Role of 9-lipoxygenase and alpha-dioxygenase oxylipin pathways as modulators of local and systemic defense. *Mol. Plant*. 5: 914–928.

11. Brodhun, F., A. Cristofal-Sarramian, S. Zabel, J. Newie, M. Hamberg, and I. Feussner. 2013. An iron 13S-lipoxygenase with an alpha-linolenic acid specific hydroperoxidase activity from Fusarium oxysporum. *PLoS One*. 8: e64919.
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