Factors Influencing the Rearrangement of *bis*-Allylic Hydroperoxides by Manganese Lipoygenase

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Running title: Rearrangement of *bis*-allylic hydroperoxides by Mn-LOX

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Abstract

Manganese lipoxygenase (Mn-LOX) catalyzes rearrangement of bis-allylic S hydroperoxides to allylic R hydroperoxides, but little is known about the reaction mechanism. 1-Linoleoyl-lysoglycerophosphatidylcholine was oxidized in analogy with 18:2n-6 at the bis-allylic carbon with rearrangement to C-13 at the end of lipoxygenation, suggesting a “tail first” model. The rearrangement of bis-allylic hydroperoxides was influenced by double bond configuration and chain length of fatty acids. The Gly316Ala mutant changed the position of lipoxygenation towards the carboxyl group of 20:2n-6 and 20:3n-3, and prevented the bis-allylic hydroperoxide of 20:3n-3 but not 20:2n-6 to interact with the catalytic metal. The oxidized form, MnIII-LOX, likely accepts an electron from the bis-allylic hydroperoxide anion with formation of the peroxyl radical, but rearrangement of 11-HPOTrE by Mn-LOX was not reduced in D2O (pD 7.5) and aqueous Fe3+ did not transfer 11S-hydroperoxy-9Z,12Z,15Z-octadecatrienoic acid to allylic hydroperoxides. Mutants in the vicinity of the catalytic metal, Asn466Leu and Ser 469Ala, had little influence on bis-allylic hydroperoxide rearrangement. In conclusion, Mn-LOX transforms bis-allylic hydroperoxides to allylic by a reaction likely based on positioning of the hydroperoxide close to Mn3+ and electron transfer to the metal with formation of a bis-allylic peroxyl radical, β-fragmentation, and oxygenation under steric control by the protein.

Supplementary key words  electron transfer, 1-linoleoyl-lysoglycerophosphatidylcholine, mass-spectrometry, metalloenzymes, peroxyl radicals, R lipoxygenase, solvent deuterium isotope effect, site-directed mutagenesis
INTRODUCTION

Lipoxygenases constitute a gene family of non-heme metalloenzymes with conserved metal ligands, which oxygenates polyunsaturated fatty acids to hydroperoxides and related compounds (1). Lipoxygenases occur in plants and mammals and are of considerable medical and biological interest (2, 3). Lipoxygenation occurs by hydrogen atom abstraction at the bis-allylic carbon of 1Z,4Z-pentadienes of fatty acids followed by O₂ insertion with formation of cis-trans conjugated (allylic) hydroperoxy fatty acids. Lipoxygenases occur in resting (reduced, Fe²⁺) and active (oxidized, Fe³⁺) forms. The active form of lipoxygenases contains the catalytic base, Fe³⁺OH (4), which forms a carbon-centered radical (L•). The latter reacts with molecular oxygen and forms a peroxyl radical:

\[
\text{Fe}^{3+} \text{OH} + \text{LH} \rightarrow \text{Fe}^{2+} \text{OH₂} + \text{L•} \quad (1)
\]

\[
\text{L•} + \text{O₂} \leftrightarrow \text{LOO•} \quad (2)
\]

In the next step, the catalytic base is regenerated with formation of the hydroperoxide:

\[
\text{LOO•} + \text{Fe}^{2+} \text{OH₂} \rightarrow \text{Fe}^{3+} \text{OH} + \text{LOOH} \quad (3)
\]

Experimental and density functional studies of the H atom abstraction by soybean lipoxygenase (sLO-1) suggest a proton-coupled electron transfer mechanism (PETC) by which the electron and the proton are transferred separately (5-7).

Autoxidation of polyunsaturated fatty acids also occur by abstraction of hydrogen at bis-allylic carbons with formation of peroxyl radicals, yet lipoxygenation and autoxidation differ in other aspects. Lipoxygenases form cis-trans conjugated hydroperoxides with stereo and position selectivity, and the initial hydrogen abstraction is associated with a large kinetic isotope effect (k_H/k_D ~40), which is due to tunneling of the deuterium atom (8). The corresponding kinetic isotope effect during autoxidation is in agreement with classical theory (k_H/k_D 5-6; (9)). Autoxidation of polyunsaturated fatty acids generates both unconjugated and cis-trans and trans-trans conjugated hydroperoxides (10-12). The spin density of the carbon centered radical is highest at the bis-allylic carbons (13) and the major products are formed by oxygenation at these positions. Rearrangement of these bis-allylic peroxyl radicals to allylic radicals occurs rapidly with a rate constant of ~2 x 10⁶, about 10⁵ times faster than rearrangement of alkyl radicals. Consequently, bis-allylic hydroperoxides only accumulate during autoxidation in the presence of high concentration
of hydrogen donors (e.g., α-tocopherol), which convert the \textit{bis}-allylic peroxy radicals to the relatively stable \textit{bis}-allylic hydroperoxides (11).

Manganese lipoxygenase (Mn-LOX) is secreted by the take-all fungus and contains Mn as the catalytic metal (14). It is the only known naturally occurring lipoxygenase, which forms significant amounts (>0.5%) of \textit{bis}-allylic hydroperoxides (15). Mn-LOX catalyzes oxygenation of 18:2n-6 by suprafacial hydrogen abstraction at C-11 and O₂ insertion at the \textit{bis}-allylic position C-11 and, with double bond migration, at the allylic position C-13. The enzyme produces 11\textit{S}-hydroperoxy-9\textit{Z},12\textit{Z}-octadecadienoic acid (11-HPODE) and 13\textit{R}-hydroperoxy-9\textit{Z},11\textit{E}-octadecadienoic acid 13-HPODE in a ~1:4 ratio (15). At the end of lipoxygenation, 11-HPODE is transformed to 13-HPODE by Mn-LOX (15). EPR analysis suggests that the catalytic metal redox cycles (Mn²⁺ / Mn³⁺) between the resting and the active form of the enzyme in analogy with Fe-lipoxygenases and that the metal coordinating residues are virtually identical (16-19).

Oxygenation of \textit{bis}-allylic carbons by Fe-lipoxygenases has so far only been reported for the recombinant lipoxygenase domain of allene oxide synthase of \textit{Plexaura homomalla}, which transforms 20:3n-6 to the \textit{bis}-allylic hydroperoxide at C-10 (~5%) and to the allylic hydroperoxide at C-8 (20). Whether the \textit{bis}-allylic hydroperoxide at C-10 was subject to rearrangement was not reported. 11-HPODE is a poor substrate of sLO-1 (15), but huge amounts of sLO-1 (36 600 U ml⁻¹, about 0.25 mg ml⁻¹) catalyze rearrangement of 11-HPODE slowly (21).

It is of interest to compare the rearrangement of \textit{bis}-allylic hydroperoxides to \textit{cis}-\textit{trans} conjugated hydroperoxides during autoxidation and Mn-LOX catalysis, as the reaction mechanisms may differ. A key intermediate is the unstable \textit{bis}-allylic peroxy radical, which transforms to an allylic peroxy radical via β-fragmentation and oxygenation at an allylic position during autoxidation (11, 12):

\begin{align*}
\text{L}_{\text{bis-allylic}}\text{OO}^• & \leftrightarrow \text{L}^• + \text{O}_2 \quad \text{(4)} \\
\text{L}^• + \text{O}_2 & \rightarrow \text{L}_{\text{allylic}}\text{OO}^• \quad \text{(5)}
\end{align*}

The LOO–H bond dissociation enthalpy (BDE) is about 88 kcal/mol, and it is likely that LOO⁺ is formed during autoxidation by hydrogen abstraction by radical species (12). Mn-LOX catalyzes rearrangement of \textit{bis}-allylic hydroperoxides likely by formation of the \textit{bis}-allylic peroxy radical, β-fragmentation, and oxygen insertion at the n-6 (or n-8) position under steric control by the enzyme.

How can \textit{bis}-allylic peroxy radicals be generated from \textit{bis}-allylic hydroperoxides
by Mn-LOX? The mechanism is likely related to the high redox potential of Mn$^{3+}$. It has been suggested that peroxyl radicals might be generated from peroxide anions by reduction of metal ions (22), suggesting the following hypothetical rearrangement mechanism for Mn-LOX:

$$
\text{Mn}^3\text{OH} + \text{L}_{\text{bis-allylic}}\text{OO}^- + \text{H}^+ \rightarrow \text{Mn}^2\text{OH}_2 + \text{L}_{\text{bis-allylic}}\text{OO}^\cdot
$$

(6)

The bis-allylic peroxyl radical is then subject to β-fragmentation and oxygen insertion under steric control by the enzyme with formation of an allylic peroxyl radical as discussed above. Recently, hypochloric acid (1 mM) was reported to convert allylic hydroperoxides of 18:2n-6 to peroxyl radicals, but the mechanism is unknown (23). In contrast, homolytic cleavage of hydroperoxides has been studied extensively. Aqueous Fe$^{2+}$, other divalent metal ions, hematin, and the reduced forms of Fe-lipoxygenases and Mn-LOX can catalyze homolytic cleavage of alkyl hydroperoxides (24, 25). Homolytic cleavage by Mn-LOX is influenced by the position of the hydroperoxide in the active site, as judged from effects of the substrate chain length and the double bond configuration (25). It seemed possible that these factors also might influence rearrangement of bis-allylic hydroperoxides to allylic.

The aim of the present study was to study factors controlling rearrangement of bis-allylic hydroperoxides by Mn-LOX. The first goal was to confirm that the fatty acids bind “tail first” in the active site of Mn-LOX during rearrangement in the same way as during oxygenation (26). The second goal was to determine the influence of the Gly316Ala mutant on the rearrangement reaction. This mutant changed the position of oxygenation and the interaction of the hydroperoxides with the catalytic metal (25). D$_2$O could be expected to increase the pK$_a$ for peroxide anions by 0.4, and this solvent deuterium isotope effect might conceivably reduce both the concentration of the anion and the rate of rearrangement. Finally, we investigated whether mutants of presumed amino acids of the first and second coordinating sphere in a hydrogen bond network might affect the rearrangement. Asn694 and Glu697 of sLO-1 belong to this group of amino acids (4) and the homologous residues of Mn-LOX are Asn466 and Ser469 (18, 19).

**EXPERIMENTAL PROCEDURES**

**Materials**

1-Palmitoyl-2-linoleoyl-GPC (99%), L$_\alpha$-lysoGPC (from soybean; 99%),
dilinoleoyl-GPC (99%), 20:2n-6 (99%), 20:3n-3 (99%) and 20:4n-6 (99%) were obtained from Larodan (Malmö, Sweden). 18:2n-6 (99%), 18:3n-6 (99%), and HPLC solvents (Lichrosolve) were from Merck. Fatty acids were dissolved in ethanol and stored in stock solutions (50-100 mM) at −20°C; fresh solutions (50-150 µM) of the fatty acids were usually prepared in 0.1 M NaBO₃ buffer (pH 9.0). Phospholipase A₂ (bee venom), cholesterol esterase (porcine pancreas) and ²H₂O (99.98%) were from Sigma-Aldrich. Site-directed mutagenesis of Mn-LOX was performed as described and the expression constructs were sequenced (19). Recombinant Mn-LOX, Mn-LOX G316A, Mn-LOX N466L, and Mn-LOX S469A were expressed in *P. pastoris* (strain X-33) as secreted proteins using the expression vector pPICZα (19). The recombinant enzymes were purified from the growth medium by hydrophobic interaction chromatography as described (14, 19), concentrated by diafiltration, and stored at +4°C.

**Mn-LOX Assay**

Light absorbance was measured with a dual beam spectrophotometer (Shimadzu UV-2101PC) with 1 cm path length. The *cis-trans* conjugated hydro(pero)xy fatty acids were assumed to have an extinction coefficient of 25 000 cm⁻¹ M⁻¹ (27). Lipoxygenase activity was monitored by UV spectroscopy (235-237 nm) in 0.1 M NaBO₃ buffer (pH 9.0) with 50-120 µM substrate. Reaction was started by addition of Mn-LO. Oxygenated fatty acids were usually analyzed after extractive isolation (SepPak/C₁₈, Waters) without acidification (19) and oxygenated 1-linoleoyl-lysoGPC after Folch extraction. The fatty acid hydroperoxides and keto fatty acids were reduced to alcohols by treatment with NaBH₄ or NaB₂H₄ before LC-MS/MS analysis unless otherwise stated. Mn-LOX was prepared in D₂O buffer by repeated diafiltration (30 k, Amicon Ultra, Millipore) and added (in <1% volume) to 65 µM 11-HPOTrE in 0.1 M NaHPO₄ (pD 7.5 and pH 7.5). pH was measured with glass electrodes from Radiometer Copenhagen. Due to the solvent isotope effect of deuterium oxide on pH glass electrodes, pD was calculated as measured pH plus 0.4 (28).

**HPLC-MS/MS**

Reversed phase-HPLC (RP-HPLC) with MS/MS analysis was performed with
Surveyor MS pump (Thermo) and with an octadecyl silica column (5 µm; 2.1x150 mm; Phenomenex or Hypersil Gold), which was usually eluted with methanol/water/acetic acid (Suprapur, Merck), 750/250/0.06, at 0.3 ml/min. The effluent passed a photodiode array detector (Surveyor PDA, 5 cm path length) and was subject to electrospray ionisation in an ion trap mass spectrometer (LTQ, Thermo). The heated transfer capillary was set at 315°C, the ion isolation width at 1.5 amu, and the collision energy at 25-35 (arbitrary scale). PGF$_{1\alpha}$ (100 ng per min) was infused for tuning. 1-Linoleoyl-lysoGPC and its metabolites were separated on the Hypersil Gold column, which was eluted with acetonitrile/5 mM ammonium acetate, 20/80, at 0.3 ml per min, with UV detection and MS/MS monitoring of positive ions.

Normal phase-HPLC (NP-HPLC) with MS/MS analysis was performed on silica with an analytical column (Kromasil-100SI; 250 x 2 mm, 5 µm, 100Å), which was eluted at 0.3 ml/min with 5% isopropanol in hexane with 0.05 ml acetic acid L$^{-1}$ (Constrametric 3200 pump, LDC). The effluent was combined with isopropanol/water (3/2; 0.2 ml/min) from a second pump (Surveyor MS pump) as described (26). The combined effluents were introduced by electrospray into the ion trap mass spectrometer (LTQ). Chiral phase-HPLC (CP-HPLC) was performed with Chiralcel OD (25x0.46 cm; Daicel through Skandinaviska GeneTech, Kungsbacka, Sweden) and eluted with 5% isopropanol in hexane with 0.1% acetic acid (0.5 ml/min). The effluent was analyzed by PDA, mixed with isopropanol/water (3/2; 0.3 ml/min) and subject to MS/MS analysis of carboxylate anions.

Miscellaneous

11-HPOTrE was isolated from incubations of 20-200 ml 0.1 M NaBO$_3$ buffer (pH 9.0) with 100 µM 18:3n-3 with ~4 nM Mn-LOX when the linear increase in UV absorbance started to deviate. The products were extracted with cold ethyl acetate, evaporated to dryness, and purified by RP-HPLC (methanol/water/acetic acid, 750/250/0.1; 4.2x100 mm, 5 µm Hypersil Gold (Thermo) or 10 µm, 8x300 mm, µBondapak C18 (Waters)), with UV detection at 210 nm and 237 nm. Fractions containing 11-HPOTrE were extracted with CH$_2$Cl$_2$, evaporated to dryness, and the amount of 11-HPOTrE was estimated by UV analysis after conversion of an aliquot to 13-HPOTrE with Mn-LOX. 1-Linoleoyl-lysoGPC was obtained by treatment of dilinoleoyl-GPC with phospholipase A$_2$,
purified by preparative TLC (CHCl₃/methanol/NH₃/H₂O, 6/3.6/0.4/0.4; Rₓ ~0.23) and characterized by RP-HPLC-MS/MS analysis. Hydrolysis of oxygenated 1-linoleoyl-lysoGPC was performed with cholesterol esterase as described (29).

RESULTS AND DISCUSSION

18:3n-3 was rapidly oxidized by Mn-LO, as shown by Fig. 1A, and the kinetic parameters (Kₘ 2.4 μM, Vₘₐₓ 17.6 μmol min⁻¹ mg⁻¹, and kₗ 2400 min⁻¹) have been reported previously (14). During the rapid linear phase of biosynthesis of UV absorbing products at 237 nm (13-HPOTrE, traces of 9-HPOTrE), the apparent amounts of the two main products were estimated by LC-MS/MS analysis. 11-HPOTrE averaged 25 % (range 22-27%) and 13-HPOTrE about 75%. The first linear increase in UV absorbance was followed by a second linear phase (at a rate of 13-15% of the first), during which 11-HPOTrE was converted to 13-HPOTrE (15). The rearrangement of 11-HPOTrE occurred slowly when low amounts of enzyme were used (4 nM Mn-LOX; Fig. 1A) and this provided a practical way to generate 11-HPOTrE.

20:4n-6 is a poor substrate of Mn-LOX and required large amounts of enzyme for rapid transformation (25, 26). Oxidation of 20:4n-6 by Mn-LOX and analysis of the products is illustrated in Fig. 1B. Analysis of products during the rapid and almost linear increase in UV absorbance at 235 nm, which is mainly due to formation of 15R-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HPETE) (26), showed that 13-hydroperoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (13-HPETE) accounted for about 10-14% of the products during the upper part of the linear phase. This number was below 1% already at the peak of UV absorbance, and <0.1% at later time points (data not shown). In comparison with 20:3n-3, the bis-allylic hydroperoxide of 20:4n-6 was rearranged rapidly.

Oxygenation of dilinoleoyl-PGC suggested that fatty acids bind the active site of Mn-LOX “tail first” (26), but it has not been confirmed whether bis-allylic hydroperoxides bind in the same way for rearrangement. Mn-LOX oxidized soybean L₀-lysoGPCs much more efficiently than 1-palmitoyl-2-linoleoyl-GPC and dilinoleoyl-GPC (Fig. 2A). Homolytic cleavage of hydroperoxides of L₀-lysoGPCs to keto fatty acids occurred at the end of lipoxygenation, as judged from a decrease in UV absorbance at 235 nm and an increase in UV absorbance with a broad maximum at 280 nm (isobestic point 259 nm). Analysis of oxygenated fatty acids after
NaBH₄ reduction and hydrolysis (0.5 M KOH in methanol) showed that 13-HODE and 13-HOTrE were formed, but 11-hydroxy metabolites could not be detected under these harsh conditions.

1-Linoleyl-lysoGPC was oxidized by Mn-LOX almost as efficiently as 18:2n-6 (Fig. 2B). LC-MS/MS analysis and UV analysis showed that 1-linoleyl-lysoGPC was oxidized by Mn-LOX to a hydroperoxy metabolite with UV absorbance maximum at 235 nm (Fig. 2C). A slightly more polar metabolite lacking significant UV absorbance at 235 nm was also formed. The MS/MS spectrum of the main metabolites ([m/z] 552 → full scan) showed signals due to sequential loss of one, two and three molecules of water ([m/z] 534 (base peak), [m/z] 516 and [m/z] 498, respectively) and possibly loss of H₂O₂ ([m/z] 518) from [M+H]+. Signals were also noted at [m/z] 452, [m/z] 434, and [m/z] 184. The MS/MS spectrum and the UV analysis were consistent with formation of a 13-hydroperoxy metabolite of 1-linoleyl-lysoGPC. *cis*-Allylic oxidation of 1-linoleyl-lysoGPC by Mn-LOX and hydroperoxide rearrangement were finally demonstrated by treatment with cholesterol esterase, which yielded a mixture of 11-HODE and 13-HODE during the linear oxidation phase but only 13-HODE at the end of lipoxygenation (Fig. 2D).

We next examined transformation of 11-HPOTrE to 13-HPOTrE by Fe³⁺. 11-HPOTrE was incubated with 10 µM Fe³⁺, and the UV absorbance at 237 nm was followed for 10 min, but it remained unchanged (Fig. 3A). The presence of 11-HPOTrE was confirmed by addition of Mn-LO, which rapidly transformed 11-HPOTrE to 13-HPOTrE as indicated in Fig. 3A. Higher concentrations of Fe³⁺ (30 and 10 µM) were also without a noticeable effect even after long incubation time. It would have been more appropriate to investigate the effect of Mn³⁺ on 11-HPOTrE. Mn³⁺ has a higher redox potential than Fe³⁺ (Mn²⁺⇔Mn³⁺ + e⁻, ~1.5 V; Fe²⁺⇔Fe³⁺ + e⁻; 0.77 V). Unfortunately, Mn³⁺ is unstable in aqueous solutions and forms MnO₂ and Mn²⁺.

The solvent deuterium isotope effect on sLO-1 kinetics has been thoroughly investigated (4, 30). Substituting H₂O with D₂O has virtually no effect on kcat of sLO-1 at room temperature (30). The solvent deuterium isotope effect on rearrangement of *cis*-allylic hydroperoxides has not been studied. Fig. 3B illustrates the rearrangement of 11-HPOTrE by Mn-LOX in 0.1 M NaHPO₄ (pH 7.5 and pD 7.5). After an initial kinetic lag phase, during which Mn-LOX likely is fully transformed to its active form by the produced 13-HPOTrE, the linear rate of oxygenation appeared to be virtually identical in the two solvents. The kinetic lag phase was not observed...
when a 1:1 mixture of 11- and 13-HPOTrE was used as substrate. The rationale for this experiment was that the pKₐ of 11-HPOTrE should increase with 0.4 in D₂O, thus reducing the concentration of the peroxide anion available for reduction by Mn³⁺-LOX. We cannot exclude that a distinct solvent isotope effect on kₗ might be present at a lower pL, but 11-HPOTrE is chemically unstable at acidic pH (31).

We next examined the effect of substrate chain length on bis-allylic hydroxylation and rearrangement. 20:2n-6 was oxygenated to 15-hydroperoxy-9Z,11E-hydroxyeicosadienoic acid (15-HEDE) as the main product and to a few percent of 11-hydroperoxy-10E,12Z-eicosadienoic acid (11-HEDE). 13-Hydroperoxy-9Z,12Z-eicosadienoic acid (13-HEDE) could not be detected by MS/MS analysis during the linear phase of oxidation.

20:3n-3 was oxidized by Mn-LOX to 13-, 11- and 15-hydroperoxyeicosatrienoic acids, as shown in Fig. 4A. The hydroxylated products were analyzed at 5 time points (Fig. 4A), and the results are shown in Fig. 4B. 15-Hydroperoxy-9Z,11E,15Z-eicosatrienoic acid (15-HETrE) accumulated as one end product. 13-Hydroperoxy-9Z,12Z,15Z-eicosatrienoic acid (13-HPETrE) was apparently rearranged to 15-HETrE, and 1-hydroperoxy-10E,12Z,15Z-eicosatrienoic acid (11-HPETrE) was also transformed to other products. NP-HPLC-MS/MS and UV analysis showed that 15-HETrE was the main product (Fig. 4C). The isomer of 15-HETrE with 9E,11E configuration could not be detected. This ruled out that 11-HPETrE was subject to hydroperoxide rearrangement.

Mn-LOX oxidizes 16:3n-3 at the n-3 position with R configuration (26), and this was also apparently the case with 11-HPOTrE. 11-HPOTrE was oxidized by Mn-LOX to two diastereoisomers of 11,18-DiHETrE, as indicated by RP-HPLC-MS/MS and UV analysis (Fig. 5A). The UV spectrum showed an absorbance maximum at 268 nm with shoulders at 260 and 279 nm, suggesting triene configuration of double bonds. The two diastereoisomers were resolved by NP-HPLC in a 2:1 ratio, as shown in Fig. 5B. The UV and MS/MS spectra of both products were identical. MS/MS analysis (m/z 337 → full scan) showed informative signals at m/z 199 (’OOC-(CH₂)₉-COH) and m/z 279 (’OOC-(CH₂)₉-CHOH-CH=CH-CH=CH-CH=CHOH), as shown in Fig. 5C. CP-HPLC-MS/MS analysis suggested that 11-HPETrE was formed as a 1:2 mixture of two stereo isomers (Fig. 5D), and both appeared to be quantitatively transformed to 11,18-DiHETrE. The elution order of the stereo isomers of 11,18-DiHETrE on NP-HPLC and 11-HETrE on CP-HPLC was not determined, but 11R-HETE elutes before 11S-
The main difference between Mn-LOX and its Gly316Ala mutant was an augmented hydroperoxide isomerase activity with formation of epoxyalcohols and keto fatty acids of 13R-hydroperoxides of 18:3n-3 and 18:2n-6, and a modest shift in the position of oxidation towards the carboxyl group (25). The G316A mutant had pronounced effects on the position of oxidation of 20:2n-6 and 20:3n-3. This mutant oxidized 20:2n-6 at all three positions, C-11, C-13 and C-15, as shown by NP-HPLC-MS/MS analysis of products during the linear increase in UV absorbance at 235 nm (cf. Fig. 6A). 13-HEDE was rearranged to 15-HEDE at the end of lipoxygenation (data not shown). The G316A mutant oxidized 20:3n-3 mainly at C-11 and to some extent at C-13 and C-15 (inset in Fig. 6B). However, the rearrangement of 13-HPETrE to 15-HPETrE, the oxygenation of 11-HPETrE to 11,18-DiHETrE, and the homolytic cleavage of 15-HPETrE to 15-keto-11Z,14E,17Z-eicosatrienoic acid appeared to be much slower than with the native enzyme, as judged from the progression curve and the MS/MS analysis (Fig. 6B and inset).

Although G316A oxidized both 20:2n-6 and 20:3n-3 at C-11, C-13 and C-15, the rearrangement of the bis-allylic hydroperoxides at C-13 thus differed.

Lipoxygenases contain three conserved residues in the sequence HxxxNxxQ. The histidin residue is an iron and manganese ligand, which is conserved along with the glutamine residue in Fe-lipoxygenases1. The Asn694 residue in this sequence of sLO-1 belongs to the first coordinating sphere, and a hydrogen bond network has been identified between Asn694 and two residues of the second coordinating sphere, Gln697 and Gln495 (4). Asn694 and Gln495 are conserved in Mn-LOX as Asn466 and Gln281, whereas Ser469 is present in the homologous position of Gln697. We assessed whether Ans466 and Ser469 of the presumed first and second coordinating spheres affected the rearrangement of bis-allylic hydroperoxides.

Mn-LOX S469A metabolized 18:2n-6, 18:3n-3, 20:2n-6 and 20:3n-3 to the same profile of metabolites as Mn-LOX. The oxidation of 20:3n-3 is shown in Fig. 6C. Mn-LOX Asn466Leu possessed only low lipoxygenase activity, as previously reported (19). 18:2n-6, and 18:3n-3 were oxidized to the same products as by native enzyme, and the 11-hydroperoxides of 18:2n-6 and 18:3n-3 were transformed to 13-hydroperoxides at the end of lipoxygenation. 20:2n-6 was transformed to 15-HEDE and ~5% 13-HEDE with
rearrangement to 15-HEDE at the end of lipoxygenation. We conclude that Gly316 is important for the interaction of 13-HPETrE with the catalytic metal, whereas neither Asn466 nor Ser469 have major effects on the rearrangement of several bis-allylic hydroperoxides.

Rearrangement of bis-allylic hydroperoxides and homolytic cleavage of hydroperoxides are likely catalyzed by Mn$^{3+}$-LOX and Mn$^{2+}$-LOX, respectively, and both reactions can be influenced by alignment of the bis-allylic and allylic hydroperoxides. The position of the bis-allylic hydroperoxide and the allylic hydroperoxide in the vicinity of the catalytic metal appears to be essential. The Gly316Ala mutant, substrate chain length and double bond configuration affected both reactions, whereas mutation of Ser469 had little effect on the homolytic cleavage of 15-HPETrE or on rearrangement of 13-HPETrE. It seems likely that Mn-LOX catalyzes direct electron transfer from the bis-allylic hydroperoxide anion to the catalytic base Mn$^{3+}$OH with subsequent generation of Mn$^{2+}$OH$_2$, the resting form of Mn-LOX. The kinetic trace for rearrangement of 11-HPOTrE to 13-HPOTrE shows a distinct kinetic lag phase in support of this mechanism.
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FOOTNOTES

Abbreviations: BDE, bond dissociation enthalpy; CP, chiral phase; GPC, glycerophosphatidylcholine; HEDE, hydroxyeicosadienoic acid; HETE, hydroxyeicosatetraenoic acid; HPEDE, hydroperoxyeicosadienoic acid; HPETe, hydroperoxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; HPOTrE, hydroperoxyoctadecatrienoic acid; LOX, lipoxygenase; Mn-LOX, manganese lipoxygenase; NP, normal phase; PCET, proton coupled electron transfer; RP, reversed phase; sLO-1, soybean lipoxygenase-1.

1The LOX-DB at www.dkfz-heidelberg.de/spec/lox-db/, as described (33).
FIGURE LEGENDS

Fig. 1. Oxygenation of 18:3n-3 and 20:4n-6 by Mn-LOX. A, progression curve with UV and LC-MS/MS analysis of products formed during oxygenation of 18:3n-3 by Mn-LOX. Mn-LOX (19 nM, 7 nM, and 4 nM) was incubated with 18:3n-3 and aliquots were analyzed for 11-HPODE and 13-HPODE, and the ratio 11-HPODE/{11-HPODE+13-HPODE} is indicated at these time points. B, oxidation of 20:4n-6 by Mn-LOX (72 nM). The bis-allylic intermediate 13-HPETE was rapidly transformed to 15-HPETE, as indicated by MS/MS analysis at two time points at the upper end of the progression curve.

Fig. 2. Progression curves for oxygenation of phosphatidylcholines by Mn-LOX to products with UV absorbance at 235 nm and LC-MS/MS analysis of products formed from 1-linoleoyl-lysoGPC. A, transformation of palmitate-linoleoyl-GPC (PLGPC), dilinoleoyl-GPC (LLGPC) and soybean Lα-lysoGPC (Lα-lysoGPC, containing about 42% 1-linoleoyl-lysoGPC and 10-15% 1-linolenoyl-lysoGPC and) to products with UV absorbance at 235 nm by Mn-LOX. B, Comparison of oxygenation of 18:2n-6 and 1-linoleoyl-lysoGPC (L-lysoGPC) by Mn-LOX (18 nM) and 50µM substrates. The direct oxidation to 13-HPODE was estimated from UV analysis (235 nm) and the relative amounts of 13-HPODE and 11-HPODE in oxidized 1-linoleoyl-lysoGPC was determined by treatment with cholesterol esterase followed by LC-MS/MS analysis. C, RP-HPLC-MS/MS analysis of dioxygenated products formed from 1-linoleoyl-lysoGPC by Mn-LOX. UV and MS/MS analysis of peak I was consistent with 1-{13-hydroperoxyoctadeca-9Z,11E-dienoate}-lysoGPC and peak II with 1-linoleoyl-lysoGPC. D, analysis of hydroxy fatty acids produced during oxidation of 1-linoleoyl-lysoGPC with Mn-LOX at the linear phase (top) and at the plateau (bottom; cf. Fig. 2B) and obtained by cleavage with cholesterol esterase.

Fig. 3. UV analysis of 11-HPOTrE treated with aqueous Fe³⁺ and with Mn-LOX in H₂O and D₂O buffers. A, 10 µM Fe³⁺ did not increase the UV absorbance at 237 nm and experiments with 30 and 100 µM yielded similar results. Mn-LOX was added at the arrow. B, kinetic traces from incubation of 65 µM 11-HPOTrE with Mn-LOX (7 nM) in 0.1 M sodium
phosphate buffer in H₂O (pH 7.5; trace a; median trace of 5 replicas) and D₂O (pD 7.5, trace b; median trace of 5 replicas).

Fig. 4. Oxygenation of 20:3n-3 by Mn-LOX. A, a kinetic trace with analysis of products at time points A-E formed from 20:3n-3 by 72 nM Mn-LOX. The solid line shows the UV absorbance at 237 nm due to biosynthesis of allylic hydroperoxides (15-HPETrE and 11-HPETrE) and the black points (A-E) mark where aliquots were taken for analysis of the oxygenated metabolites by LC-MS/MS. B, LC-MS/MS analysis of 11-, 13- and 15-HETrE at time points A-E after reduction with NaBH₄. C, NP-HPLC-MS/MS analysis of products formed during the linear increase of UV absorbance (top) and at the end of lipoxygenation (bottom).

Fig. 5. RP-HPLC- and NP-HPLC-MS/MS analysis of 15-HETrE, 11-HETrE and 11,18-DiHETrE at the end of lipoxygenation. A, RP-HPLC-MS/MS analysis of products. Top - total ion current of MS/MS (m/z 321 → full scan); the main peak with a retention time of 13 min contained 15-HETrE. Middle - reconstructed ion chromatogram of the characteristic fragment of 11-HETrE during MS/MS analysis (m/z 321 → m/z 199) magnified 10 times. Bottom - total ion current of MS/MS analysis (m/z 337 → full scan); the main peak contained 11,18-DiHETrE. B, separation of diastereoisomers of 11,18-DiHETrE by SP-HPLC and analysis by MS/MS (m/z 337 → full scan) and by UV (268 nm). D, reconstructed ion chromatogram for steric analysis of 11-HETrE by CP-HPLC-MS/MS with monitoring of the characteristic signal at m/z 199. MS/MS analysis showed that peaks marked I and II contained 11-HETrE.

Fig. 6. Oxygenation of 20:2n-6 and 20:3n-3 by Mn-LOX G316A and 20:3n-3 by S469A. A, NP-HPLC-MS/MS analysis of products formed from 20:2n-6 by G316A. The oxygenation at C-11 and C-13 is insignificant by the native enzyme, which illustrates that oxygenation was shifted towards the carboxyl group. B, Oxidation of 20:3n-3 by G316A. The oxygenation is shifted towards C-13 and C-11 (inset with MS/MS analysis top and UV analysis at 237 nm bottom). The rearrangement of 13-HPETE to 15-HPETE appeared to be blocked, as product analysis at the indicated three time-points yielded virtually
identical amounts of 11-, 13-, and 15-HETE, and there were no indication of a significant homolytic cleavage of 15-HPETE (no decline at UV absorbance at 237 nm). C, oxygenation of 20:3n-3 by S469A. The insert shows the relative amounts of hydroxy metabolites during the initial phase of oxygenation. The kinetic trace appeared to be identical to that of the native enzyme. 13, 11 and 15 denotes 13-H(P)ETE, 11-H(P)ETE and 15-H(P)ETE in the insets of B and C.
FIGURES

Fig. 1A

![Graph showing absorbance over time](image1)

Fig. 1B

![Graph showing absorbance over time with peaks](image2)
Fig. 2A

![Graph with absorbance over time](image)

Fig. 2B

![Graph with absorbance over time](image)
Fig. 2C

- TIC
- MS/MS 552
- mAU (235 nm)

Retention Time (min)

Fig. 2D

- Relative Abundance

Retention Time (min)
Fig. 4C

Fig. 5A
Fig. 5B

Fig. 5C
Fig. 5D

![Fig. 5D](chart1)

Fig. 6A

![Fig. 6A](chart2)