A G316A Mutation of Manganese Lipoxygenase Augments Hydroperoxide Isomerase Activity

**MECHANISM OF BIOSYNTHESIS OF EPOXYALCOHOLS**

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Mirela Cristea and Ernst H. Olw

From the Department of Pharmaceutical Biosciences, Biomedical Center, Uppsala University, SE-751 24 Uppsala, Sweden

Lipoxygenases with R stereospecificity have a conserved Gly residue, whereas (S)-lipoxygenases have an Ala residue. Site-directed mutagenesis has shown that these residues control position and S/R stereospecificity of oxygenation. Recombinant Mn-LO was expressed in *Pichia pastoris*, and its conserved Gly-316 residue was mutated to Ala, Ser, Val, and Thr. The G316A mutant was catalytically active. We compared the catalytic properties of Mn-LO and the G316A mutant with 17:3n-3, 18:2n-6, 18:3n-3, and 19:3n-3 as substrates. Increasing the fatty acid chain length from C17 to C19 shifted the oxygenation by Mn-LO from the n-6 toward the n-8 carbon. The G316A mutant increased the oxygenation at the n-8 carbon of 17:3n-3 and at the n-10 carbon of the C17 and C18 fatty acids (from 1–2% to 7–11%). The most striking effect of the G316A mutant increased the oxygenation at the carbon of 17:3, 18:3, and 17:3n-3, respectively, to keto fatty acids and epoxyalcohols. The n-3 double bond was an essential. An experiment under an oxygen-18 atmosphere showed that both oxygen atoms were retained in the epoxyalcohols. (R)-Hydroperoxides at n-6 of C17:3, 18:3, and 19:3 were transformed 5 times faster than S stereoisomers. The G316A mutant converted (13R)-hydroxyeicosatetraenoic acid to 13-ketotetraenoic acid (with an apparent *Km* of 0.01 mM) and to epoxyalcohols (viz. erythro- and threo-11-hydroxy-(12R,13S)-epoxy-9Z,15Z)-octadecadienoic acids and one of the corresponding cis-epoxides as major products). A reducing lipoxygenase inhibitor stimulated the hydroperoxide isomerase activity, whereas a suicide-type lipoxygenase inhibitor reduced this activity. The n-3 double bond also appeared to influence the anaerobic formation of epoxyalcohols by Mn-LO, since 18:2n-6 and 18:3n-3 yielded different profiles of epoxyalcohols. Our results suggest that the G316A mutant augmented the hydroperoxide isomerase activity by positioning the hydroperoxy group at the n-6 carbon of n-3 fatty acids closer to the reduced catalytic metal.

Lipoxygenases are iron- or manganese-containing dioxygenases, which are widely spread in nature (1). They oxidize polyunsaturated fatty acids by hydrogen abstraction at the *cis*-allylic carbon of the (12Z,4E)-pentadiene, leading to biosynthesis of *cis-trans* conjugated and *cis*-allylic hydroperoxy fatty acids (1–3). All lipoxygenases belong to the same gene family, which is characterized by sequence homology and by conserved metal ligands (1, 4, 5). Iron lipoxygenases occur in animals and plants, whereas manganese lipoxygenase has been demonstrated in *Gaeumannomyces graminis*, the take-all fungus of wheat (6), and homologous genes occur in the rice blast fungus, *Magnaporthe grisea*, and in *Aspergillus fumigatus* (XP_362938 and XP_746463, respectively, reported in GenBank™, available on the World Wide Web at www.ncbi.nlm.nih.gov).

Lipoxygenases have important biological functions. In animals, lipoxygenases catalyze oxidation and degradation of cellular organelles (7, 8). In plants, lipoxygenases may take part in the chemical warfare between plants, fungi, and other pathogens (9). Lipoxygenases participate in biosynthesis of signal molecules from hydroperoxides in animals and plants (e.g. to leukotrienes, lipoxins, traumatin, jasmonic acid, epoxyalcohols (hepoxilins), and related oxylipins) (1, 9–11).

Fatty acid hydroperoxides can be isomerized to epoxyalcohols and keto fatty acids by epoxyalcohol synthases, cytochrome P450, and other enzymes and by diverse catalysts (e.g. by cysteine-FeCl₃, hemoglobin, and hematín) (12–16). Lipoxygenases may also transform hydroperoxides to epoxyalcohols and keto fatty acids under conditions of limiting oxygen supply or high substrate concentration or in the presence of reducing agents (17–20).

The biological importance of the transformation of hydroperoxy fatty acids to epoxyalcohols was highlighted with the discovery of mutated genes for eLOX₃ and for arachidonate (12R)-LOX in patients with two disorders of skin keratinization, nonbullous congenital ichthyosiform erythroderma and congenital ichthyosis (21–23). eLOX₃ is classified as a member of the lipoxygenase gene family, but it lacks lipoxygenase activity (11). eLOX₃ converts (12R)-HPETE to an allylic epoxyalcohol, (8R)-hydroxy-(11R,12R)-epoxy-(5Z,9E,14Z)-eicosatrienoic acid, and to 12-KETE, but it also transforms other hydroperoxides of arachidonic acid (11).

The three-dimensional protein structures are available for sLO-1 and -3, rabbit reticulocyte (15S)-LOX, and coral (8R)-LOX (24–28). Single or sequential mutations of residues of the substrate binding cavity have extensively been studied, since they affect substrate positioning and may change the position specificity of the enzyme, from one end of the (1Z,4Z)-pentadiene to the other (29–37). Coffa and Brash (38) observed that lipoxygenases, which form hydroperoxides with S chirality, have a conserved Ala residue (or, occasionally, a Ser residue), whereas human and mouse (12R)-LOXs and coral (8R)-LOX have a conserved Gly residue.

**References**

1. To whom correspondence should be addressed: Division of Biochemical Pharmacology, Dept. of Pharmaceutical Biosciences, Uppsala University, P.O. Box 591, SE-751 24 Uppsala, Sweden. Tel.: 46-184714455; Fax: 46-18552936; E-mail: Ernst.Olww@farmbio.uu.se.

2. See also the lipoxygenase data base LOX-DB, available on the World Wide Web at www.dkfz-heidelberg.de/spec/lox-db/(54).

3. The abbreviations used are: eLOX₃, epidermal lipoxygenase-3; BW A4C, N-(3-phenoxynamyl)acetohydroxamic acid; ETYA, eicosatetraynoic acid; HODE, hydroxyoctadecadienoic acid; HETE, hydroperoxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatrienoic acid; HODE, hydroperoxyeicosatrienoic acid; HETE, hydroperoxyeicosatrienoic acid; HPETE, hydroperoxyeicosatrienoic acid; HODE, hydroperoxyeicosatrienoic acid; HETE, hydroperoxyeicosatrienoic acid; HPETE, hydroperoxyeicosatrienoic acid; HETE, hydroperoxyeicosatrienoic acid; KETE, ketocis-tetraenoic acid; KETE, ketocis-tetraenoic acid; KETE, ketocis-tetraenoic acid; LC, liquid chromatography; mass spectrometry with MS/MS facility; Mn-LO, manganese lipoxygenase; RP, reversed phase; LOX, lipoxygenase; SP, straight phase; TPP, triphenylphosphine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; sLO, soybean lipoxygenase.
TABLE 1
The Coffa and Brash determinant (Ala/Gly) of S and R stereospecificity of six lipoxygenases and an alignment with Mn-LO and eLOX3

| Position | Sequence | Name   | GenBank™ accession |
|----------|----------|--------|--------------------|
| 412      | INTLARELIL | Human (15S)-LOX-2 | U78294 |
| 413      | INTLARELLIV | Mouse (85)-LOX | AK028724 |
| 538      | INL9QRGLL | sLO-1 | P08170 |
| 437      | INSTGREAVILL | Human (12R)-LOX | AF038461 |
| 437      | INSIGRALL | Mouse (12R)-LOX | NP_033789 |
| 447      | VIN3G1KALL | Coral (8R)-LOX | U59223 |
| 311      | HPSMV0LRNL | Mn-LO | AAK81882 |
| 448      | VNTIARALIL | eLOX3 | BCI0508 |

idude in the corresponding position (Table 1). Mutation of Ala to Gly of two (S)-lipoxygenases (human (15S)-LOX-2 and mouse (85)-LOX) altered the chirality of oxygenation to R and also the position specificity to the other end of the (1Z,4Z)-pentadiene, whereas the Gly to Ala mutation of two (R)-lipoxygenases (human (12R)-LOX and coral (8R)-LOX) (Table 1) changed the position and chirality to S in the same fashion (38). These results have now been extended to mutations of sLO-1 and murine (12R)-LOX (37, 39). The results could be explained by a model where the Gly and Ala residues change the substrate position relative to an oxygen access channel, leading to antarafacial oxygen insertion at either end of the (1Z,4Z)-pentadiene (40, 41). This oxygen channel appears to be narrow, since Gly to Val mutations of murine and human (12R)-lipoxygenases and Ala to Val mutations of three (S)-lipoxygenases led to loss of enzymatic activity (37, 39). Whether Ala/Gly also affects the hydroperoxidase activity of eLOX3 (Table 1) has not yet been reported.

Mn-LO differs from all iron lipoxygenases in one important aspect; Mn-LO catalyzes suprafacial oxygenation, whereas iron lipoxygenases have been repeatedly found to catalyze antarafacial oxygenation (2). An explanation of this phenomenon could be that the oxygen access channel to the reactive pentadiene differs between Mn-LO and other lipoxygenases.

The amino acid sequence of Mn-LO can be aligned with 23–27% identity to plant and to mammalian S- and R-lipoxygenases, and the metal ligands are conserved (4, 5). Mn-LO also contains the conserved Gly-316 residue of (R)-lipoxygenases (Table 1). Mn-LO catalyzes R lipoxygenation, but the alignments do not suggest a higher degree of amino acid identity with (R)-lipoxygenases than with (S)-lipoxygenases. The preferred substrate of Mn-LO is α-linolenic acid, which is oxidized at the n-6 carbon to (11S)-HPOTe and the n-6 carbon to (13R)-HPOTe, and the latter accumulates as the end product (2, 6). The oxygenation of the bis-allenic carbon and hydroperoxide migration from C-11 to C-13 constitute an unprecedented feature of this lipoxygenase. Whether chain shortening of 18:3n-3 to 17:3n-3 or chain elongation to 19:3n-3 affects the oxidations of the n-6 and n-8 carbons have not been investigated.

Our first aim was to study the catalytic importance of Gly-316. We hypothesized that the different oxygenation mechanism and possible structural differences in oxygen access channels between Mn-LO and iron lipoxygenases might be revealed by mutation of Gly-316 to Ala, Val, Ser, and Thr residues, since these mutations of other (R)-lipoxygenases have been investigated (37, 38). Our second aim was to compare the oxygenation of 17:3n-3, 18:3n-3, 18:2n-6, and 19:3n-3 by Mn-LO and its mutants.

We report that mutation of Gly-316 to Ala in Mn-LO changed the oxygenation of 17:3n-3 and 18:3n-3 essentially as predicted by Coffa and Brash (37–39, 41), although the effects were less marked than anticipated. The other three mutations were catalytically silent. We found that the G316A mutation increased the hydroperoxide isomerase activity of (R)-hydroperoxides at the n-6 position of 19:3n-3, 18:3n-3, and 17:3n-3. As a mechanism, we propose that the G316A mutation shifts the position of the hydroperoxide group at the n-6 carbon closer to the reduced catalytic metal and that an n-3 double bond is essential to provide necessary structural rigidity by interaction with π-electrons (42). Our results suggest that the Gly/Ala residues at position 316 of Mn-LO control the position of the n-3 fatty acids in relation to the oxygen insertion channel and the distance of the hydroperoxide group at n-6 to the catalytic metal.

EXPERIMENTAL PROCEDURES

Materials—18:2n-6 (99%) and 18:3n-3 (99%) were from Merck, and 17:3n-3 (99%) and 19:3n-3 (99%) were from Larodan (Malmö, Sweden). The fatty acids were dissolved in ethanol and stored in stock solutions (50–100 mM) at −20 °C. Fresh solutions (50–100 μM) of the fatty acids in 0.1 M NaOH buffer (pH 9.0) were prepared daily. (11S- and (13R)-hydroperoxides of 18:2n-6 and 18:3n-3 and (R)-hydroperoxides at n-6 of 17:3n-3 and 19:3n-3 were prepared by biosynthesis using Mn-LO as described (3). (S)-Hydroperoxides at n-6 of 17:3n-3, 18:2n-6, 18:3n-3, and 19:3n-3 were prepared by sLO-1 (lipoxygenase type IV; Sigma) (3). (9S)-HPODE and (9S)-HPOTe were prepared by tomato lipoxygenase (43). 13-KODE, 13-KOTe, methyl (9S)-hydroxy-(10R,11E)-epoxy-(9Z)-octadecenoate, methyl (9S)-hydroxy-(10S,11S)-epoxy-(9Z)-octadecenoate, methyl (11R)-hydroxy-(12S,13S)-epoxy-(9Z)-octadecenoate, methyl (11S)-hydroxy-(12S,13S)-epoxy-(9Z)-octadecenoate, methyl (13S)-hydroxy-(11R,12R)-epoxy-(9Z)-octadecenoate, methyl (13S)-hydroxy-(11S,12S)-epoxy-(9Z)-octadecenoate, and oxygen-18 (99.5%) werefrom Larodan. Saponification was performed chemically (0.5 M KOH in 90% methanol; 70 °C, 1 h under argon) or biologically with rat blood plasma (alloxygenal epoxycarboxylic acids; 50 μg hydrolyzed with 80 μl of rat plasma, 0.1 M sodium phosphate buffer (pH 7.4) (1:1); 90 min at 21 °C under argon). ETYA was from Hoffman-La Roche (Basel, Switzerland), BW A4C was from the Wellcome Foundation (Beckenham, UK), and norphydroxyaacidic acid was provided by Dr. Hamberg (Karolinska Institutet). Bovine hemin, NaBH₄, NaBH₄, TPP, sorbitol, and glass beads (0.5 mm) were from Sigma. pPICZαA, Pichia pastoris (strain X-33), Zeocin, yeast nitrogen base, competent Escherichia coli (Top 10), and DNA ladders were from Invitrogen. PCR primers and HPLC-purified oligonucleotides for site-directed mutagenesis were obtained from CyberGene (Huddinge, Sweden) and TIB MolBiol (Berlin, Germany). Tag and Pfu polymerases and the QuikChange kit for site-directed mutagenesis were from Stratagene. Yeast extract, peptone from soybeans, most organic salts, and solvents (analytical grade or higher quality) were from Merck. Centrifugation filters (Amicon Ultra-15) and cartridges with octadecyl silica (SepPak/C₁₈) were from Waters. Sequencing was performed by cycle sequencing using ABI Prism BigDye terminator from PerkinElmer Life Sciences at Uppsala Genomic Center (Rudbeck Laboratories, Uppsala University). Buffers were prepared with water from Milli-Q plus 185 (Waters). Equipment for SDS-PAGE was from Bio-Rad (6).

Expression and Site-directed Mutagenesis—Recombinant Mn-LO was expressed in P. pastoris and secreted into the growth medium (buffered minimal methanol) using an expression construct with the alcohol oxidase promoter (pPICZαA-Mn-LO-602) and with the native secretion signal replaced with the yeast secretion α signal, as described (5). Site-directed mutagenesis of pPICZαA-Mn-LO-602 was performed with oligonucleotides of 42 nucleotides and Pfu polymerase as described (5). The PCR mixture was treated with DpnI for 3 h, checked for amplification of the target plasmid by agarose gel electrophoresis, and used to transform E. coli by electroporation. Plasmid DNA was isolated and sequenced. Pichia cells were transformed as described (5), and recombi-
nents were selected by Zeocin resistance (5). Genomic DNA was isolated from resistant colonies and screened for incorporation of the Mn-LO construct by PCR (forward, 5'-TGGATCTGACGTACAGCCCCTCGA; reverse, 5'-ATCCAGTTCTTGAGCTTAGTCG; annealing at 55 °C) with equipment as described (5). We also confirmed by sequencing that the *Pichia* colonies used for expression contained the construct with the desired mutation.

**Enzyme Purification**—Mn-LO was purified to homogeneity as described (5). Mn-LO G316A, G316E, G316V, and G316T were purified from the buffered minimal methanol medium (0.3–0.5 liters of *Pichia* cultures in baffled flasks). After 5–6 days of methanol induction, the A<sub>600</sub> reached ~15, and the cells were precipitated by centrifugation. Ammonium sulfate was added to the supernatant to 0.6 M, and the pH was adjusted to 7.0 (10 M NaOH). After centrifugation, the supernatant was loaded on a phenyl-Sepharose column (6). Bound proteins were eluted with low salt buffer and concentrated to ~1 ml by diafiltration. The expression of the recombinant proteins was checked by SDS-PAGE as described (5) and identified by MALDI-TOF analyses of tryptic peptides, which were formed by in gel digestion (44).

**Enzyme Assay**—Lipoxygenase activity was monitored by UV spectroscopy (at 235 and 237 nm; 10-mm path length cuvettes of 0.15, 0.5, or 2 ml) in 0.1 M sodium borate buffer (pH 9.0), usually with 50–100 μM fatty acid substrates and 2–15% enzyme solution (by volume). Products were analyzed after extractive isolation (SepPak/C<sub>18</sub> or CH<sub>2</sub>Cl<sub>2</sub>) and in some experiments reduced with NaBH<sub>4</sub>, NaB<sub>2</sub>H<sub>4</sub>, or 1–10 M NaBO<sub>3</sub> buffer was repeatedly evacuated with an oil pump and flushed with argon. Under anaerobic conditions, we added 50 mM NaBO<sub>3</sub> buffer, pH 9.0, was incubated under normal atmosphere with 0.1 M NaBO<sub>3</sub> buffer was repeatedly evacuated with an oil pump and flushed with argon. Under anaerobic conditions, we added 50 mM NaBO<sub>3</sub> buffer, pH 9.0, and the reaction was terminated after 60 min with 2 ml of ethanol, diluted with 12 ml of water, and immediately extracted (SepPak/C<sub>18</sub>).

**Aerobic Incubation**—First, (13R)-HPD<sub>O</sub> and (13R)-HPOT<sub>E</sub> were generated in situ. Recombinant Mn-LO (50 μg) in 5 ml of 0.1 M NaBO<sub>3</sub> buffer, pH 9.0, was incubated under normal atmosphere with 100 μM 18:2n-6 or 18:3n-3 until complete conversion of substrate had occurred (UV analysis). The vessel was then sealed, and the atmosphere was repeatedly evacuated with an oil pump and flushed with argon. Under anaerobic conditions, we added 50 μg of Mn-LO (in 0.5 ml of 0.1 M NaBO<sub>3</sub> buffer, pH 9.0) and 18:2n-6 or 18:3n-3 to a final concentration of 100 μM. The reaction proceeded for 1.5 h (21 °C). It was terminated by the addition of methanol and followed by extractive isolation (14).

**Epoxycalcohols**—Epoxycalcohols were prepared from (13R)-HPD<sub>E</sub>, (11S)-HPD<sub>E</sub>, (9S)-HPD<sub>E</sub>, (13R)- and (13S)-HPOT<sub>E</sub>, and (9S)-HPOT<sub>E</sub> (40–50 μg) in 0.2 ml of 0.1 M K<sub>5</sub>HPO<sub>4</sub> by treatment with 13–26 μg of hematin (dissolved in 0.01 M NaOH) and collected after 10 min with CH<sub>3</sub>Cl<sub>2</sub> (13, 14). Epoxycalcohols were also generated by incubating SLO-1 with 18:2n-6 or 18:3n-3 in 0.1 M NaBO<sub>3</sub> (pH 9.0) with 2–20 μM hematin, followed by extractive isolation.

**HPLC Analysis**—Separation of methyl 13-HODE and methyl 9-HODE were performed by SP-HPLC (Nucleosil 50–5, 250 × 4.6 mm; 2 ml/min) and by chiral phase HPLC ([R]–(–)-N,3,5-dinitrobenzoxyl-o-phenylglycine, 250 × 4.1 mm; 0.8 ml/min) with 0.5% isopropyl alcohol in hexane (45). The system contained a diode array detector (Waters 996 PDA) and pump (CM-4000, Milton Roy). The RP-HPLC columns (Kromasil 5 C<sub>18</sub> (250 × 2 × 5 μm, 10 Å; Phenomenex) and Hypersil Gold (150 × 2 mm; 5 μm; Thermo) were eluted with methanol/water/acetic acid (80:20:0.01 at 0.3–0.4 ml/min (P2000, SpectroSystem) for analysis of hydroxy fatty acids, whereas a 70:30:0.01 mixture was used for partial separation of epoxycalcohols. SP-HPLC analysis of epoxycalcohols was performed on a Kromasil-100 column (250 × 2 × 5 μm, 100 Å), which was eluted at 0.3 ml/min (ConstaMetric 3200; LDC Analytical) with 1 or 3% isopropyl alcohol in hexane (with 0.1–0.03 ml/liter acetic acid) for analysis of hydroxy fatty acids and epoxycalcohols, respectively. To avoid daily variations in retention times on SP-HPLC, the flow rate was usually adjusted so that three-11-hydroxy-(12S,13S)-epoxy-(9Z)-octadecenoate had a retention time of ~12 min.

**LC-MS Analysis**—RP-HPLC with electrospray ionization was performed with an ion trap mass spectrometer (LCQ; Thermo) as described (46). The heated capillary was set to 215 °C. In order to obtain ionization in the electrospray process, the SP-HPLC effluent (0.3 ml/min) was mixed with isopropyl alcohol/water (6/4; 0.2 ml/min) in a T junction and introduced to the mass spectrometer. An LTQ ion trap (Thermo) was used with a photodiode array detector (Surveyor, Thermo) in the later part of this study.

**Spectroscopy**—Light absorbance was measured with a dual beam spectrophotometer (Shimadzu UV-2101PC). The cis-trans conjugated hydro(pero)xy fatty acids were assumed to have an extinction coefficient of 25,000 cm<sup>–1</sup> M<sup>–1</sup> (5).

**RESULTS**

**Catalytic Properties of Mn-LO G316A:** Linoleic Acid—Mn-LO G316A metabolized 18:2n-6 to (11S)-HPD<sub>E</sub> and (13R)-HPD<sub>E</sub> in approximately the same relative amounts as the native enzyme, and (13R)-HPD<sub>E</sub> accumulated as the end product. MS/MS analysis revealed that significant but small amounts of 9-HPD<sub>E</sub> also were formed (signal at m/z 171 ([M–(CH<sub>3</sub>)<sub>2</sub>CHO]– appearing in the LC-MS/MS spectrum of 13-HODE due to co-eluting 9-HODE). Relative to (13R)-HODE, the native enzyme formed ~2% (9S)-HPD<sub>E</sub> and the mutant 7–10% (9S)-HPD<sub>E</sub> as judged by SP-HPLC and chiral phase HPLC analysis. Hydroperoxide isomerase activity with (13R)-HPD<sub>E</sub> as a substrate was insignificant compared with (13R)-HPOT<sub>E</sub> (see below).

**α-Linolenic Acid**—Mn-LO G316A metabolized 18:3n-3 to (13R)-HPOT<sub>E</sub> and (11S)-HPOT<sub>E</sub>, and the G316A mutant also metabolized (11S)-HPOT<sub>E</sub> to (13R)-HPOT<sub>E</sub> in analogy with Mn-LO.

The kinetic UV trace (237 nm) showed that Mn-LO G316A also converted (13R)-HPOT<sub>E</sub> to products with less UV absorption at this wavelength (Fig. 1A). To study this phenomenon, we compared the catalytic properties of the G316A mutant with recombinant Mn-LO, using 100 μM 18:3n-3 as a substrate and similar amounts of enzyme, as judged from the initial rate of linear biosynthesis of cis-trans conjugated products. The native enzyme oxidized the substrate to completion after a kinetic time lag. After a similar kinetic time lag, Mn-LO G316A increased (13R)-HPOT<sub>E</sub> formation linearly (maximal rate of 0.46
absorbance units/min at 100 μM 18:3n-3), and it leveled off after consumption of ~75% of the substrate (after 5 min), and the amount of (13R)-HPOTrE then declined at a rate of 0.08 absorbance units/min. UV analysis showed that the decrease in absorbance at 237 nm was accompanied by an increase in absorbance at 282 nm with an isosbestic point at 252 nm (Fig. 1, inset). As described below, this was due to hydroperoxide isomerase activity of G316A.

Authentic 13-KOTrE has a λmax at 282 nm (ethanol), and LC-MS/MS analysis confirmed that 13-KOTrE was formed along with epoxyalcohols. The G316A mutant transformed (13R)-HPOTrE to 13-KOTrE at a linear rate and without apparent time lag. Apparent K_m for the isomerization of (13R)-HPOTrE to 13-KOTrE was ~10 μM. Heat-inactivated Mn-LO G316A transformed (13R)-HPOTrE to 13-KOTrE in insignificant amounts (1 h, 21 °C).

Comparison with Mn-LO—Using 0.09–0.7 μM enzyme, it was possible to demonstrate that the native enzyme also possessed low but detectable hydroperoxide isomerase activity. A comparison of the hydroperoxide isomerase activities of Mn-LO and the G316A mutant with 18:3n-3 as a substrate is shown in Fig. 1B by following the decline in (13R)-HPOTrE. The decline in UV absorption was at least 7-fold faster with G316A. The transformation of (13R)-HPDE by Mn-LO was negligible in comparison with (13R)-HPOTrE (Fig. 1C). The n-3 double bond thus appeared to be essential for the hydroperoxide isomerase activity. As a percentage of the maximal lipoxygenase activity, the hydroperoxide isomerase activities of Mn-LO and the G316A mutant were ~1% and 7–8%, respectively.

17:3n-3 and 19:3n-3—Mn-LO seemed to oxygenate 50 μM 17:3n-3 and 18:3n-3 with the same maximal rates (Fig. 2A). The kinetic traces were also similar, but the products differed. 17:3n-3 was converted by Mn-LO mainly to 12-HPHTrE (oxidation at n-6), and only traces of 10-HPHTrE (oxidation of n-8) were detected during the linear part of the lipoxygenation curve (MS/MS analysis). The kinetic traces suggested that the relative rate of the hydroperoxide isomerase activity was slightly reduced with 17:3n-3 as a substrate in comparison with 18:3n-3 (Fig. 2B).

The G316A mutation affected the oxidation of 17:3n-3 in two ways. First, the rate of lipoxygenation was decreased, and the hydroperoxide isomerase activity was increased in comparison with 18:3n-3 (Fig. 2B). The G316A mutant transformed 17:3n-3 to both 12-HPHTrE (~93%) and 10-HPHTrE (~7%) during the linear phase (MS/MS analysis). The G316A mutant thus partly shifted oxygenation from the n-6 toward the n-8 carbon. The MS/MS analysis also revealed an increased oxygenation to 8-HPHTrE (from 1–2 to 11% in analogy with (9S)-HPODE above), but this was not further investigated.

19:3n-3 was a relatively poor substrate in comparison with 18:3n-3, but its kinetic trace showed several distinct features (Fig. 2C). A short kinetic time lag was followed by a relatively rapid and linear increase in UV absorbance at 237 nm (for 1–2 min), followed by a slower increase (for 2 min) and then a steady decline in UV absorbance (at the same rate as the decay of (13R)-HPOTrE). As a percentage of the maximal lipoxygenase activity, the hydroperoxide...
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The G316A mutant yielded a different kinetic trace with a monophasic linear increase in UV absorbance. This was followed first by a rapid decline (in comparison with 18:3 \(n\)-3) and then a slow decline (Fig. 2 D).

LC-MS/MS analysis showed that Mn-LO first transformed 19:3 \(n\)-3 by oxidation at the \(n\)-8 carbon (to 12-HPNTrE) with less oxidation at the \(n\)-6 carbon (to 14-HPNTrE). In the initial phase (2 min), the relative amounts were 12-HPNTrE/14-HPNTrE/14-KNTrE (Fig. 3), but 2 min later, 14-KNTrE had accumulated as the main product with approximately equal amounts of 12- and 14-HPNTrE (data not shown). We expected G316A to favor oxidation at the \(n\)-8 carbon relative to the \(n\)-6 carbon, but LC-MS/MS analysis showed that 12-HPNTrE was less abundant than 14-HPNTrE during the linear phase.

Effect of Lipoxygenase Inhibitors—ETYA and BW A4C inhibit Mn-LO (6). We found that ETYA (100 \(\mu\)M), a suicide-type inhibitor (47), reduced the rate of conversion of (13R)-HPOTrE to 13-KOTrE by Mn-LO G316A by 75% (Fig. 4 A). BW A4C (100 \(\mu\)M) is a metal-reducing lipoxygenase inhibitor and augmented the initial transformation rate of (13R)-HPOTrE to 13-KOTrE severalfold (Fig. 4 A), but the drug then slowed down and blocked the reaction after a few min (cf. Ref. 48). This inhibitory effect was less pronounced when smaller amounts of enzyme were used.

The stimulatory effect of BW A4C on the hydroperoxide isomerase activity was concentration-dependent, as illustrated in Fig. 4 B, with a severalfold increase in the initial rate of transformation of (13R)-HPOTrE to 13-KOTrE at 30–100 \(\mu\)M BW A4C, but the stimulatory effect declined at 300 \(\mu\)M BW A4C. For comparison, 100 \(\mu\)M ETYA was superior.

FIGURE 2. Lipoxygenase and hydroperoxide isomerase activities of Mn-LO and Mn-LO G316A with 17:3 \(n\)-3 and 19:3 \(n\)-3 as substrates. A, transformation of 18:3 \(n\)-3 and 17:3 \(n\)-3 by Mn-LO. B, transformation of 18:3 \(n\)-3 and 17:3 \(n\)-3 by G316A. C, transformation of 19:3 \(n\)-3 and 18:3 \(n\)-3 by Mn-LO. D, transformation of 19:3 \(n\)-3 by Mn-LO and by G316A.

The stimulatory effect of BW A4C on the hydroperoxide isomerase activity was concentration-dependent, as illustrated in Fig. 4 B, with a severalfold increase in the initial rate of transformation of (13R)-HPOTrE to 13-KOTrE at 30–100 \(\mu\)M BW A4C, but the stimulatory effect declined at 300 \(\mu\)M BW A4C. For comparison, 100 \(\mu\)M ETYA was superior.

FIGURE 3. LC-MS/MS analysis of products formed from 19:3 \(n\)-3 by Mn-LO at the initial phase of lipoxygenation. Top, MS/MS analysis (m/z 305 → full scan) of 12-KNTrE. Bottom, MS/MS analysis (m/z 305 → full scan) of 12-HNTrE (peak I) and 14-HNTrE (peak II). The products were isolated after a 2-min incubation of 100 \(\mu\)M 19:3 \(n\)-3 with 0.5 \(\mu\)M Mn-LO, and hydroperoxides were reduced to alcohols with TPP. The products were separated RP-HPLC and analyzed by an ion trap mass spectrometer (LTQ). NL, normalized ion intensity of LTQ.

FIGURE 4. Effect of lipoxygenase inhibitors and stereochemistry of the hydroperoxyl group on the hydroperoxide isomerase activity of Mn-LO G316A. A, time curve for the conversion of (13R)-HPOTrE (50 \(\mu\)M) to 13-KOTrE and the effect of ETYA (100 \(\mu\)M) and BW A4C (100 \(\mu\)M). The graphs show UV absorbance at 280 nm. B, effect of 3–300 \(\mu\)M BW A4C on the rate of transformation of (13R)-HPOTrE to 13-KOTrE relative to the rate without inhibitor. C, relative rates of conversion of \(R\) and \(S\) 12-HPHTrE, \(R\) and \(S\) 13-HPOTrE, and \(R\) and \(S\) 14-HPNTrE, respectively, to keto compounds (UV absorbance at 280 nm).

The stimulatory effect of BW A4C on the hydroperoxide isomerase activity was concentration-dependent, as illustrated in Fig. 4 B, with a severalfold increase in the initial rate of transformation of (13R)-HPOTrE to 13-KOTrE at 30–100 \(\mu\)M BW A4C, but the stimulatory effect declined at 300 \(\mu\)M BW A4C. For comparison, 100 \(\mu\)M ETYA was superior.
BW A4C only inhibited the lipoygenase reaction by ~40% in these experiments.

Nordihydroguaiaretic acid (100 μM) did not inhibit the lipoygenase activity of the G316A mutant (or Mn-LO), and the drug had no effect on the initial rate of transformation of (13R)-HPOTrE, but it appeared to reduce the conversion of (13R)-HPOTrE after 7–8 min in analogy with this late effect of BW A4C (data not shown).

Transformation of R- and S-Hydroperoxides of n-3 Fatty Acids—Mn-LO G316A was incubated with the (R)- and (S)-hydroperoxides at n-6 of 17:3n-3, 18:3n-3, and 19:3n-3. The (R)-hydroperoxides were transformed to keto compounds at a rate that was 5 times higher than for the corresponding S-hydroperoxides (Fig. 4C). We confirmed by LC-MS/MS analysis that 19:3n-3 and 17:3n-3 also were converted to epoxycarboxylic acids.

**Product Identification**—The products formed from 18:3n-3 by Mn-LO G316A were studied by LC-MS/MS (Fig. 5). RP-HPLC showed that Mn-LO G316A formed 11-HPOTrE and 13-HPOTrE in the same ratio as recombinant Mn-LO during the linear lipoygenation phase (with a modest increase in 9-HOTrE as discussed above; data not shown). With time, 13-KOTrE and polar products accumulated. 13-KOTrE (peak 5 in Fig. 5A) was identified by UV analysis and by MS/MS analysis of the carboxylate anion (A-; m/z 291 → full scan), which showed signals, inter alia, at m/z 273 (A-18), m/z 247 (A-44, loss of CO2, 100%), m/z 179, m/z 177, and m/z 167. Authentic 13-KOTrE showed the same MS/MS spectrum.

Reduction of the products with NaB2H4 yielded 13-HOTrE with significant deuterium incorporation at C-13, whereas the deuterium incorporation of the corresponding 11- and 9-hydroxy-(12Z,13Z)-hydroperoxides at C-13 was minimal in the Mn-LO G316A reaction. Therefore, differences in the retention times of authentic 13-KOTrE and 13-HOTrE were observed (Fig. 5A).

The products formed by Mn-LO G316A after incubation with 18:3n-3 for 50 min were separated by SP-HPLC and analyzed by MS/MS as shown in Fig. 5, B and C. 13-KOTrE eluted as the least polar product. To simplify the analysis, hydroperoxides were reduced to alcohols with TBP, and the MS/MS spectrum of 12-HOTrE showed a characteristic signal at m/z 211 (~OOC-(CH2)7-CH=CH-CH2-HCO). (13R)-HPOTrE was shown to be almost completely converted to products by Mn-LO G316A and it was present in smaller amounts than both 11-HOTrE and 9-HOTrE.

The most polar products were separated as shown in Fig. 5C. Based on systematic analysis of hematin-catalyzed isomerization of fatty acid hydroperoxides to epoxyalcohols discussed below and on authentic standards, the two major products were identified as erythro- and threo-11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid (peaks 1 and 2), which were formed in a ratio of 2:3. In addition to these two trans-epoxides, the corresponding erythro- and threo-isomers of the corresponding cis-epoxide also appeared to be present as minor products (peaks 3 and 4). The MS/MS analysis and the retention time of the material in peak 5 was consistent with an isomer of 9-hydroxy-(12S,13R)-epoxy-(10E,15Z)-octadecadienoic acid.
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![Diagram](image)

**FIGURE 6.** MS/MS spectra of epoxyalcohols. A, MS/MS analysis (m/z 309 → full scan) of 11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid. B, MS/MS analysis (m/z 309 → full scan) of 9-hydroxy-(12R,13R)-epoxy-(10E,15Z)-octadecadienoic acid. The spectra were magnified ×10 as indicated.

**A**

**B**

differentiated, m/z 247 (265-18), m/z 227 (12OOC-(CH2)7-CHO), m/z 211 (12OOC-(CH2)7-CH=CH-CH(OH)-CHO), m/z 199, m/z 197 (12OOC-(CH2)7-CH=CH-CHO) and m/z 219, m/z 181, and m/z 169 and in the lower mass range at m/z 153 (197-44) and m/z 111 (Fig. 6A). MS/MS analysis of the allylic epoxyalcohol, 9-hydroxy-(12R,13R)-epoxy-(10E,15Z)-octadecadienoic acid, showed strong signals at m/z 291 (A1-18), m/z 273 (A2-2 × 18), m/z 265 (A4-44), m/z 247 (A4-44), m/z 227 (12OOC-(CH2)7-CH=CH-CH=CHO), and weaker signals at m/z 211 (12OOC-(CH2)7-CH=CH-CH=CH-CHO), m/z 209 (227-18), m/z 193 (211-18), m/z 183, m/z 171 (possibly 12OOC-(CH2)7-CH=CH, CH2), m/z 165 (209-44), and m/z 137 (12OOC-(CH2)7-CH=CH-C2H4), as shown in Fig. 6B.

**Incorporation of Oxygen-18**—Incubation of Mn-LO G316A under oxygen-18 atmosphere with 18:3n-3 yielded labeled erythro and threo isomers of 11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid in a ratio of 2:3. MS/MS analysis (m/z 313 → full scan) yielded intense signals, inter alia, at m/z 295 (313-18, loss of water from the carboxyl group), m/z 293 (313-20, loss of 18O2), m/z 269 (313-44), m/z 231 (227 + 4; 12OOC-(CH2)7-CH=CH-CH(18O)-CH=CH2) and at m/z 199 (197 + 2; 12OOC-(CH2)7-CH=CH-CH(18O)-CH=CH2) with virtually no signal at m/z 197. MS/MS analysis (m/z 311 → full scan) revealed essentially no formation of 11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid with one oxygen-18. These results were consistent with essentially complete incorporation of oxygen-18 in the hydroxyl group at C-11 and in the epoxide at C-12-13 and showed that the mutant Mn-LO G316A possessed hydroperoxide isomerase activity.

**Effect of Oxygen Tension**—We also investigated the effect of saturated oxygen concentration (1.1 mM O2) on the hydroperoxide isomerase activity. In oxygen-saturated buffer, the maximal linear rate of biosynthesis of (13R)-HPOTrE was increased, but it leveled off after a few min. The UV absorption at 237 nm then declined at a rate 25% lower than in a parallel experiment with Mn-LO G316A under normal oxygen tension (0.22 mM O2).

G316S, G316T, and G316V–G316T and G316V were catalytically inactive, as reported for the corresponding mutations of (12R)-lipoxygenases and (S)-lipoxygenase (37, 39), and this was also the case with G316S (assayed with 17:3n-3, 18:3n-3, 19:3n-3, and (13R)-HPOTrE). The expression levels of these mutants were similar to the G316A mutant and expression was confirmed by digestion of the SDS-PAGE band and MALDI-TOF analysis with identification of tryptic peptides of Mn-LO.

**Anaerobic Incubation of Mn-LO: Linoleic Acid**—Products formed by Mn-LO during anaerobic incubation with (13R)-HPOTrE and 18:2n-6 were analyzed by RP-HPLC and by SP-HPLC. They were derived mainly from (13R)-HPOTrE with significant contribution from both (11S)-HPOTrE and (9S)-HPOTrE. RP-HPLC with MS/MS analysis showed that epoxyalcohols were formed along with (11S)-HODE, (13R)-HODE, 13-KODE, and small amounts of 11-KODE with MS/MS spectra as reported (46).

The epoxyalcohols were separated by SP-HPLC as shown in Fig. 7A. Erythro- and three-13-hydroxy-trans-11,12-epoxy-(9Z)-octadecenoic acid (formed from (11S)-HPOTrE) and erythro- and three-11-hydroxy-trans-12,13-epoxy-(9Z)-octadecenoic acid (formed from (13R)-HPOTrE) exhibited very similar MS/MS spectra but could be identified by their retention times on SP-HPLC. This also applies to the 9-hydroxy-(10S,11S)-epoxy and 11-hydroxy-(9S,10S)-epoxy compounds formed from (11S)-HPOTrE and (9S)-HPOTrE, respectively.

The three major products (peaks 1, 3, and 6 in Fig. 7A) appeared to have the same retention times as erythro- and three-11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecenoic acid and as a cis-epoxy, 11-hydroxy-(12S,13R)-epoxy-(9Z)-octadecenoic acid, respectively. In addition, a series of minor products were noted. The material in peak 4 showed strong signals at m/z 201 and m/z 171, and it was identified as three-9-hydroxy-(10S,11S)-epoxy-(12Z)-octadecenoic acid by its retention time and by MS/MS analysis. LC-MS/MS analysis of peaks 7 and 8 showed strong and characteristic signals at m/z 193, and the MS/MS spectra were consistent with isomers of 9-hydroxy-(12S,13R)-epoxy-(10E)-octadecenoic acid. The material in peaks 2 and 5 had the same retention times and MS/MS spectra as erythro- and three-11-hydroxy-(12R,13S)-epoxy-(9S,10S)-epoxy-(12Z)-octadecenoic acid. We conclude that (13R)-HPOTrE and 18:2n-6 are mainly transformed to erythro- and three-11-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid and one cis-epoxy isomer (11-hydroxy-(12S,13R)-epoxy-(9Z)-octadecenoic acid) under anaerobic conditions. As expected, epoxyalcohols derived from (11S)-HPOTrE and (9S)-HPOTrE were formed as minor products.

**α-Linolenic Acid**—Anaerobic incubation of Mn-LO with (13R)-HPOTrE and 18:3n-3 yielded a different pattern of epoxyalcohols than with (13R)-HPOTrE and 18:2n-6 (Fig. 7B). The major product in peak 1 was identified as erythro-11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecenoic acid (by retention time and MS/MS spectrum). In addition, small amounts of the three isomer also appeared to be formed (peak 2). The MS/MS spectrum of the material in peak 3 was consistent with an isomer of 11-hydroxy-(12S,13R)-epoxy-(9Z,15Z)-octadecenoic acid, whereas the MS/MS spectrum of the material in peak 4 was consistent with an isomer of 9-hydroxy-(12S,13R)-epoxy-(10E,15Z)-octadecenoic acid. The MS/MS spectrum of the material in peak 5 was not consistent with available standards. The least polar products consisted mainly of 13-KOTrE.

**Comparison with Hematin-catalyzed Transformations**—We used the hematin-catalyzed transformation of hydroperoxides to generate epoxyalcohol standards and to compare this nonenzymatic formation with biosynthesis by Mn-LO G316A and with formation by Mn-LO under anaerobic conditions.
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FIGURE 7. SP-HPLC-MS/MS analysis of epoxyalcohols formed by Mn-LO during anaerobic conditions and epoxyalcohols formed by hematin-catalyzed transformation. A, products formed from (13R)-HPODE and linoleic acid anaerobically. See “Anaerobic Incubation of Mn-LO: Linoleic Acid” under “Results” for details. Peaks 1, 3, and 6 contained erythro- and threeo-11-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid and 11-hydroxy-(12S,13R)-epoxy-(9Z)-octadecenoic acid, respectively, and peaks 4, 7, and 8 contained threeo-9-hydroxy-(10S,11S)-epoxy-(12Z)-octadecenoic acid and two isomers of 9-hydroxy-(12S,13R)-epoxy-(10E)-octadecenoic acid, respectively. Peaks 2 and 5 contained erythro- and threeo-11-hydroxy-(9S,10S)-epoxy-(12Z)-octadecenoic acid. B, products formed from (13R)-HPOTrE and α-linolenic acid anaerobically. Peak 1 contained erythro-11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid, whereas threeo-11-hydroxy-(12R,13R)-epoxy-(9Z,15Z)-octadecadienoic acid eluted in peak 2. Peak 3 probably contained an isomer of 11-hydroxy-(12R,13S)-epoxy-(10E,15Z)-octadecadienoic acid. The material in peak 5 was not consistent with available standards. C, epoxyalcohols formed from (13R)-HPODE by hematin treatment. Peaks 1 and 2 contained erythro- and threeo-11-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid, respectively, whereas one of the corresponding cis-epoxides (11-hydroxy-(12S,13D)-epoxy-(9Z)-octadecenoic acids) eluted in peak 3. Peak 4 mainly contained (9R)-hydroxy-(12R,13R)-epoxy-(10E)-octadecenoic acid, and peak 5 mainly contained (9S)-hydroxy-(12R,13R)-epoxy-(10E)-octadecenoic acid (cf. Ref. 49). The material in peaks 6 and 7 probably consisted of the corresponding cis-epoxides (9R-hydroxy-(12R,13S)-epoxy-(10E)-octadecenoic acids). D, epoxyalcohols formed from (13S)-HPOTrE by hematin treatment. Peak 1 and 2 contained erythro- and threeo-11-hydroxy-(12S,13S)-epoxy-(9S,15S)-octadecadienoic acid, respectively, whereas the corresponding cis-epoxides (11-hydroxy-(12S,13R)-epoxy-(9S,15S)-octadecadienoic acids) eluted in peaks 3 and 4. The material in peaks 5 and 6 probably consisted of (9R)-hydroxy- and (9S)-hydroxy-(12S,13S)-epoxy-(10E,15S)-octadecadienoic acids with the corresponding cis-epoxides eluting in peaks 7 and 8. * and **, peaks that contained material with the same MS/MS spectra. In A–D, the normalized ion intensities (1.3E7 etc.) refer to analysis by the LCQ ion trap mass spectrometer. In A and C, 13-KODE was a major product, which eluted after 5.8 min (not shown), whereas 13-KODE eluted after 5.9 min in B and D (data not shown).

(13R)-HPODE—Hematin-catalyzed transformation yielded 13-KODE and epoxyalcohols, as outlined in Fig. 8, in agreement with previous studies (13, 14).

The epoxyalcohols were separated by SP-HPLC as shown in Fig. 7C into two pairs of major products (peaks 1, 2, 4, and 5). MS/MS analysis and comparison with authentic standards showed that peaks 1 and 2 contained erythro- and threeo-11-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid, respectively, with identical MS/MS spectra. Peaks 4 and 5 were identified as (9R)- and (9S)-hydroxy-(12R,13S)-epoxy-(10E)-octadecenoic acid, respectively, by their relative retention times on SP-HPLC (49) and by MS/MS analysis, yielding practically identical mass spectra. 13-KODE eluted after 5.8 min, and the MS/MS spectrum (m/z 329 → full scan) showed signals, inter alia, at m/z 275 (A − 18, 35%), m/z 249 (A − 44, 100%), m/z 195 (~15%), m/z 179 (~12%, possibly with loss of H₂C═C(OH)═CH₂, ~35%), m/z 177 (~40%), and m/z 167 (25%), confirmed by MS/MS analysis of authentic 13-KODE.

Small amounts of the corresponding cis-epoxides were also formed (14). The LC-MS/MS analysis suggested that peaks 3 and 4 contained the erythro and three isomers of 11-hydroxy-(12S,13R)-epoxy-(9Z)-octadecenoic acid, and the latter might thus co-elute with (9S)-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid, as judged from the reconstructed ion chromatogram (single reaction monitoring, m/z 311 → m/z 197). The minor products of peaks 6 and 7 showed identical MS/MS spectra with (9R)- and (9S)-hydroxy-(12R,13R)-epoxy-(10E)-octadecenoic acid and were probably the corresponding cis-epoxides.

On RP-HPLC the elution order was reversed. Erythro- and threeo-11-hydroxy-(12R,13R)-epoxy-(9Z)-octadecenoic acid were partially separated and appeared to elute after 24.1 and 22.8 min, respectively. The two 9-hydroxy-(12R,13R)-epoxy-(10E)-octadecenoic acids eluted after 19.4 and 20.3 min, and the corresponding cis-epoxides eluted in small peaks after 16.4 and 15.7 min (data not shown).

(13S)-HPOTrE—Hematin-catalyzed transformation yielded 13-KOTrE and epoxyalcohols. The latter were partly separated by SP-HPLC as shown in Fig. 7D in analogy with the separation of (13R)-HPODE-derived products (Fig. 7C). The four major compounds were thus identified by MS/MS analysis and retention times on SP-HPLC as erythro- and threeo-11-hydroxy-(12S,13S)-epoxy-(9Z,15Z)-octadecadienoic acid in peaks 1 and 2, respectively, and (9S)- and (9R)-hydroxy-(12S,13S)-epoxy-(10E,15Z)-octadecadienoic acids in peaks 5 and 6, respectively. The minor products were
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FIGURE 8. Summary of major products formed during hematin-catalyzed transformation of (13R)-HPODE and (11S)-HPODE. Transformation of (13R)-HPODE is shown on the left, and (11S)-HPODE is shown on the right. Compounds 1 and 2 are epoxyalcohols, whereas compound 3 to the left is 13-KODE and to the right 11-KODE.

probably the corresponding cis-epoxides (viz. erythro- and threo-11-hydroxy-(12Z,13S)-epoxy-(9Z,15Z)-octadecadienoic acid (peaks 3 and 4) and (9S)-hydroxy- and (9R)-hydroxy-(12R,13S)-epoxy-(10E,15Z)-octadecadienoic acids (peaks 7 and 8).

On RP-HPLC, erythro- and threo-11-hydroxy-(12S,13S)-epoxy-(9Z,15Z)-octadecadienoic acid co-eluted in a broad major peak after 16 min, and the corresponding cis-epoxides co-eluted after 11–13 min in a minor peak. The allenic epoxides, 9-hydroxy-12,13-epoxy-(10E,15Z)-octadecadienoic acids, eluted after 9 min.

(11S)-HPODE—Hematin-catalyzed transformation of (11S)-HPODE yielded, as expected, 11-KODE and erythro and threo isomers of 9-hydroxy-(10S,11S)-epoxy-(12Z)-octadecenoic acid and erythro and threo isomers of 13-hydroxy-(11R,12R)-epoxy-(9Z)-octadecenoic acid as major products (outlined in Fig. 8) and small amounts of the corresponding cis-epoxides.

SP-HPLC with MS/MS analysis of products formed from (11S)-HPODE suggested that erythro- and threo-13-hydroxy-(11R,12R)-epoxy-(9Z)-octadecenoic acid eluted after 10.4 and 11.7 min, respectively, and erythro- and threo-9-hydroxy-(10R,11R)-epoxy-(12Z)-octadecenoic acid after 12.1 and 15.2 min, respectively. The LC-MS/MS analysis was in agreement with the corresponding authentic standards.

Co-injections showed that erythro-9-hydroxy-trans-10,11-epoxy-(12Z)-octadecenoic acid eluted on the left shoulder of erythro-11-hydroxy-trans-12,13-epoxy-(9Z)-octadecenoic acid, whereas threo-9-hydroxy-trans-10,11-epoxy-(12Z)-octadecenoic acid eluted after threo-11-hydroxy-trans-12,13-epoxy-(9Z)-octadecenoic acid.

(9S)-HPODE and (9S)-HPOTrE—Since these hydroperoxides are formed as minor products by Mn-LO and Mn-LO G316A, we also prepared the corresponding epoxyalcohols as standards. Epoxyalcohols derived from (9S)-HPODE were identified in anaerobic incubation of Mn-LO. The MS/MS spectra of erythro- and threo-11-hydroxy-(9S,10S)-epoxy-(12Z)-octadecenoic acid (derived from (9S)-HPODE) were practically identical to those of erythro- and threo-9-hydroxy-(10S,11S)-epoxy-(12Z)-octadecenoic acids (derived from 11-HPODE), but their retention times differed. The fact that these regioisomeric epoxyalcohols show identical MS/MS spectra is probably due to epoxide migration (Pyanne rearrangement) in the gas phase of the mass spectrometer (51). erythro-11-Hydroxy-(9S,10S)-epoxy-(12Z)-octadecenoic acid eluted between threo- and threo-11-hydroxy-trans-12,13-epoxy-(9Z)-octadecenoic acid, and threo-11-hydroxy-(9S,10S)-epoxy-(12Z)-octadecenoic acid eluted after threo-9-hydroxy-(10S,11S)-epoxy-(12Z)-octadecenoic acid on NP-HPLC.

DISCUSSION

We report that site-directed mutagenesis of a single amino acid, G316A, in the active site of Mn-LO augmented its hydroperoxide isomerase activity severalfold and slightly shifted the position of oxygenation from the n-6 toward the n-8 and n-10 carbons. Mn-LO and its mutant catalyzed four reactions: hydrogen abstraction, oxygen insertion, migration of hydroperoxides at the n-8 carbon to the n-6 carbon (peroxide rearrangement), and transformation of n-6 hydroperoxides to epoxyalcohols and keto compounds. A discussion of these reactions would benefit from a hypothetical model where the substrate fatty acids enter the hydrophobic catalytic cavity with their methyl ends and with their carboxyl groups anchored, by ionic interaction, to a charge residue at a fixed position (28, 42). The n-8 carbon should be close to the catalytic metal to allow lipxygenation.

The first and rate-limiting step in Mn-LO catalysis is abstraction of the pro-S hydrogen at the n-8 carbon by the catalytic base, Mn$^{3+}$OH (2). Mn-LO oxidized 17:3n-3 and 18:3n-3 at similar rates, whereas 19:3n-3 was oxidized slowly. The distance from the catalytic metal to the pro-S hydrogen at the n-8 carbon appeared to be essential for hydrogen abstraction and might contribute to the low turnover of 19:3n-3. The π-electron interaction of the n-3 double bonds with aromatic amino acids probably orients these substrates with their n-8 carbons closer to the catalytic base, since both 18:2n-6 and 18:3n-6 are oxidized less efficiently than 18:3n-3 (6). The G316A mutant reduced the rate of oxygenation of 17:3n-3 in comparison with 18:3n-3, suggesting that the distance of n-8 carbon of 17:3n-3 to the catalytic base was increased. Whether the G316A mutation affected the absolute rate of lipxygenation in comparison with Mn-LO will await further studies.

In the next step, the carbon-centered radical at the bis-allylic n-8 carbon reacts with molecular oxygen. The spin density is largest at the n-8 carbon (52), but oxygen insertion is controlled by steric factors. 17:3n-3 is oxidized by Mn-LO almost exclusively at the n-6 carbon (to 12-HPHTrE), whereas chain elongations to 18:3n-3 shifted oxygenation to 70–80% at the n-6 carbon (to 13R)-HPOTrE and 20–30% at the n-8 carbon (to (11S)-HPOTrE) and chain elongations to 19:3n-3 shifted oxygenation to 20–30% at the n-6 carbon (to (14R)-HPNTrE) and 70–80% at the n-8 carbon (to (12S)-HPNTrE). Based on the model discussed above, these results suggest that the oxygenation channel only...
allowed access to the n-6 carbon of 17:3n-3 and to both the n-6 and n-8 carbons of 18:3n-3 and 19:3n-3.

Mn-LO G316A positioned some fatty acids so that the oxygen channel had access also to the n-8 and the n-10 carbons. The oxidation of 17:3n-3 shifted from almost exclusively at the n-6 carbon toward the n-8 (7%) and n-10 (11%) carbons. The oxidation of 18:3n-3 by Mn-LO G316A was also shifted toward n-10 carbon with an increased formation of (9S)-HPOTrE. Under anaerobic conditions, Mn-LO formed erythro-11-hydroxy(12R,13S)-epoxy-(9Z,15Z)-octadecadienoic acid as the main epoxyalcohol. R denotes the side chain of 18:3n-3 with C-1 to C-8.

The relative formation of hydroperoxides at the n-6 and n-8 carbons can also be affected by migration of hydroperoxides at n-8 to n-6 (cf. Fig. 9), since the n-6 hydroperoxides accumulate as the end products (2). During the linear phase, Mn-LO thus transforms 18:3n-3 to (13R)-HPOTrE and (11S)-HPOTrE in a ratio of ~3:4:1. The concentration of the substrate declines, (11S)-HPOTrE is converted to (13R)-HPOTrE in a less efficient process than the peroxyl oxygenation. The kinetic analysis of 19:3n-3 suggested a similar mechanism. Rapid transformation of 19:3n-3 by Mn-LO to (12S)-HPNTrE and (14R)-HPNTrE with consumption of the substrate was followed by migration of the hydroperoxide group from n-8 to n-6 to yield the end product, (14R)-HPNTrE, at a lower rate.

The most striking effect of the G316A mutation was the increased hydroperoxide isomerase activity. The major products formed by Mn-LO G316A with α-linolenic acid as a substrate are summarized in Fig. 9. The mutant efficiently catalyzed homolytic cleavage of the hydroperoxide of (13R)-HPOTrE with formation of an alkoxy radical at C-13. The alkoxy radical was transformed to erythro and threo isomers of 11-hydroxy(12R,13S)-epoxy-(9Z,15Z)-octadecadienoic acid in a 2:3 ratio and to 13-KOTrE as the major products. We also noted formation of cis-epoxides of 11-hydroxy-12,13-epoxy-(9Z,15Z)-octadecadienoic acid and 9-hydroxy-(12R,13S)-epoxy-(10E,15Z)-octadecadienoic acid as minor products. The mutant G316A thus formed two major trans-epoxyalcohols (the erythro and threo isomers), whereas the pseudoperoxidase hydroperoxide isomerase activity of Mn-LO (and Mn-LO G316A). The chain length also influenced the reaction. Mn-LO exhibited approximately the same and relatively low hydroperoxide isomerase activity toward (14R)-HPNTrE and (13R)-HPOTrE, whereas (12R)-HPNTrE appeared to be transformed less efficiently than (13R)-HPOTrE.

Mn-LO G316A transformed (14R)-HPNTrE, (13R)-HPOTrE, and (12R)-HPNTrE ~2, 7, and 15 times more efficiently than Mn-LO. The mutation G316A presumably moved the hydroperoxide groups closer to the catalytic metal. The G316A mutant transformed (14R)-HPNTrE, (13R)-HPOTrE, and (12R)-HPNTrE about 5 times faster than the corresponding hydroperoxides with S stereo configuration, suggesting that the hydroperoxides with R configuration are positioned closer to the catalytic metal.

It is of interest to compare the reaction mechanism of Mn-LO G316A with eLOX3, which has been characterized in detail (11). Both enzymes catalyze retention of the original hydroperoxide oxygens in the epoxyalcohols in hydroperoxide isomerase reactions but form different
epoxyalcohols. eLOX3 transformed (12R)-HPETE to (8R)-hydroxy-
(11R,12R)-epoxyicosatetraenoic acid and 12-KETE and transformed
(15S)-HPETE to 16R-13-hydroxy-(14S,15S)-epoxyicosatrienoic acid
and 15-KETE and (12S)-HPETE to at least two epoxyalcohols and to
12-KETE (11). eLOX3 was stimulated ~4-fold by nortylohydroxuaraetic acid,
which probably reduces Fez+ to Fe2+, whereas a hydroxamic acid
derivative (BW A4C) stimulated the hydroperoxide isomerase activity
of Mn-LO G316A ~5-fold, presumably by reducing Mn3+ to Mn2+ (20).
BW A4C inhibited lipoxigenation by the same mechanism. In contrast
to eLOX3, nortylohydroxuaraetic acid did not stimulate the
hydroperoxide isomerase activity of Mn-LO G316A, and the drug did
not block its lipoxynase activity.

In summary, the reduced catalytic metals initiate homolytic cleavage
of the hydroperoxide oxygen-oxygen bond of the fatty acid and form
an alkoxyl radical and Mn3+OH and Fe3+OH, respectively. The alkoyl
radical rearranges to a keto fatty acid and to a trans-epoxide with a
carbon-centered radical, to which oxygen is rebound with formation
of epoxyalcohols (or reduced to water with formation of keto fatty acids).
In this step, the catalytic metal is reduced to Mn2+ and Fe2+, and a new
hydroperoxide isomerase cycle may begin, as outlined in Fig. 10.

What prevents some lipoxynases from catalyzing efficient hydro-
peroxide isomerase reactions under normal oxygen tension in analogy
with eLOX3 and Mn-LO G316A? A reason may be related to the
redox potential of the catalytic metal and to steric factors. The redox
potentials (eel, V) are as follows: Fe3+ + ee− → Fe2+ (+0.77), and
Mn3+ + ee− → Mn2+ (+1.51). Mn3+ is thus more difficult to oxidize than Fe2+.
Brash and co-workers (11) proposed that the ferric redox
state of eLOX3 was unfavorable. The stable ferrous form made eLOX3
incapable of performing abstraction of (12R)-LOX cf.

Hydroperoxide Isomerase Activity

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