Catalytic Convergence of Manganese and Iron Lipoxygenases by Replacement of a Single Amino Acid

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Background: 13R-MnLOX catalyzes suprafacial hydrogen abstraction and oxygenation in contrast to sLOX-1.

Results: Mutation of one residue of 13R-MnLOX altered hydrogen abstraction and oxygenation to antarafacial.

Conclusion: Replacement with the corresponding residue of soybean LOX-1 yielded catalytic convergence.

Significance: The suprafacial oxygenation mechanism can be attributed to a single amino acid substitution.

Lipoxygenases (LOXs) contain a hydrophobic substrate channel with the conserved Gly/Ala determinant of regio- and stereospecificity and a conserved Leu residue near the catalytic non-heme iron. Our goal was to study the importance of this region (Gly332, Leu336, and Phe337) of a lipoxygenase with catalytic manganese (13R-MnLOX). Recombinant 13R-MnLOX oxidizes 18:2n-6 and 18:3n-3 to 13R-, 11(S or R)-, and 9S-hydroperoxy metabolites (~80–85, 15–20, and 2–3%, respectively) by suprafacial hydrogen abstraction and oxygenation. Replacement of Phe337 with Ile changed the stereochemistry of the 13-hydroperoxy metabolites of 18:2n-6 and 18:3n-3 (from ~100% R to 69–74% S) with little effect on regiospecificity. The abstraction of the pro-S hydrogen of 18:2n-6 was retained, suggesting antarafacial hydrogen abstraction and oxygenation. Replacement of Leu336 with smaller hydrophobic residues (Val, Ala, and Gly) shifted the oxidation from C-13 toward C-9 with formation of 9S- and 9R-hydroperoxy metabolites of 18:2n-6 and 18:3n-3. Replacement of Gly332 and Leu336 with larger hydrophobic residues (G332A and L336F) selectively augmented dehydration of 13R-hydroperoxoctadeca-9Z,11E,15Z-trienoic acid and increased the oxidation at C-13 of 18:1n-6. We conclude that hydrophobic replacements of Leu336 can modify the hydroperoxide configurations at C-9 with little effect on the R configuration at C-13 of the 18:2n-6 and 18:3n-3 metabolites. Replacement of Phe337 with Ile changed the stereospecific oxidation of 18:2n-6 and 18:3n-3 with formation of 13S-hydroperoxides by hydrogen abstraction and oxygenation in analogy with soybean LOX-1.

Lipoxygenases (LOXs) are fatty acid dioxygenases with a non-heme catalytic metal, usually iron (1–3). They oxidize polyunsaturated fatty acids with 1Z,4Z-pentadiene units to hydroperoxides, which are precursors to biological mediators in mammals, plants, and fungi (2, 4, 5). In humans, LOX products are formed from oxidation of arachidonic acid, and these eicosanoids interact with G-protein-coupled receptors; modulate asthma and allergic inflammation, atherosclerosis, and skin water permeability; and contribute to oxidative stress and cancer development (4, 6–8). The versatile role of LOX metabolites has led to the development of enzyme inhibitors and receptor antagonists (4). Plant LOX pathways oxygenate C19 fatty acids to volatile jasmonates, aldehydes, and a series of other metabolites (9). These oxylipins participate in the chemical warfare between plants and microorganisms or herbivores and act as signals in plant development (2, 5, 9, 10).

All LOXs belong to the same gene family, which is characterized by sequence homology of their catalytic domains and by conserved iron ligands (1, 3, 11–13). Crystal structures are available for several soybean LOXs (sLOXs), coral 8R-LOX, arachidonate 15- and 5-LOX, and one prokaryotic LOX (14–19). The binding of fatty acids to the active sites has so far with one recent exception (18) been deduced by modeling, site-directed mutagenesis, and oxidation of fatty acids with different chain lengths and numbers of double bonds (16, 20–25).

LOXs abstract the bisallylic hydrogen from C-3 of the 1Z,4Z-pentadiene of unsaturated fatty acids followed by insertion of molecular oxygen at C-1, C-5, or occasionally C-3 with biosynthesis of hydroperoxy fatty acids (26, 27); the lipoxidation of 18:2n-6 is outlined in Fig. 1A. Recently, a U-shaped substrate channel of LOX was proposed (16). In this model based on the coral 8R-LOX crystal structure, the carboxyl group of fatty acids binds to one of the two channel entrances with the ω-end embedded in the protein (16). The two opposite orientations will allow hydrogen abstraction from C-3 of the 1Z,4Z-pentadiene with antarafacial oxygen insertion at either C-1 (e.g. 8R-LOX) or at C-5 (e.g. sLOX-1) (16). Three of the four conserved metal ligands are His residues, and the fourth is the carboxyl of the C-terminal amino acid, usually Ile. Two of these His residues are found in a specific sequence hexamer (His-(Leu/Trp)-(Leu/(Asn/Arg)-(Thr/Gly)-His). The iron also binds water and interacts with a distant Asn or His residue, forming an octahedral ligand configuration (3). The metal-water complex
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constitutes the catalytic base (Fe$^{3+}$OH$^{-}$) for hydrogen abstraction and redox cycling.

13R-MnLOX of *Gaemumannyces graminis* and 9S-MnLOX$^{3}$ of *Magnaporthe salvinii*, fungal root and stem pathogens of wheat and rice, respectively, differ from FeLOX in three aspects (28). First, the metal-coordinating spheres are not identical. The manganese ligands are likely three His residues, the carboxylate of the C-terminal amino acid residue, and an Asn residue in analogy to iron ligands of FeLOX (11, 29), but the hexamer motif of FeLOX is truncated to a pentamer, His-Val-Leu-Phe-His, in MnLOX (11). Second, MnLOX oxidizes hydroperoxides to peroxyl radicals at a rate almost 2 orders of magnitude higher than that of sLOX-1 (30). The mechanism is unknown but could be related to the metal centers. The redox potentials of Mn$^{2+}/3+$ and Fe$^{2+}/3+$ differ by a factor of 2, which is reflected in the catalysis of organic Mn- and FeLOX mimics (30, 31). Third, 13R- and 9S-MnLOX$^{3}$ catalyze suprafacial hydrogen abstraction and oxygenation, whereas sLOX-1 and presumably all FeLOXs catalyze antarafacial hydrogen abstraction and oxygenation (26) (see Fig. 1A). How can this catalytic difference be explained?

9S- and 13R-MnLOX and sLOX-1 likely bind 18:2n-6 in the same “head-tail” orientation at pH 9, and all three enzymes abstract the pro-S hydrogen at C-11 and form a pentadienyl radical (26, 32).$^{3}$ The same faces of the 9Z and 12Z double bonds of this radical are shielded from oxygen insertion by 9S- and 13R-MnLOX, respectively, whereas the other side of the 12Z double bond is shielded from oxygen in sLOX-1 (see Fig. 1A). Linoleic acid is thus likely positioned so that hydrophobic residues protect opposite faces of the 12Z double bond from oxygen insertion in 13R-MnLOX and sLOX-1. We lack detailed structural information on the active sites of 9S- and 13R-MnLOX, but it seems likely that many structural features of FeLOX are conserved in the vicinity of the catalytic manganese. It is therefore conceivable that minor structural changes could contribute to the unique oxidation mechanism of MnLOX. In this region, a conserved Leu of FeLOX is believed to position the 1Z,4Z-pentadiene close to the catalytic iron (16, 33). Replacements of these Leu residues of sLOX-1 and coral 8R-LOX and the adjacent Ile residue of coral 8R-LOX have been investigated and found to reduce or even abolish catalysis (16, 33). This Leu residue is conserved in 13R- and 9S-MnLOX.$^{3}$ The adjacent Phe residue is characteristic of 9S- and 13R-MnLOX as all other LOXs have Ile or Val residues at this position (see Fig. 1B).

We therefore hypothesized that Leu$^{336}$, Phe$^{337}$, and the pentamer motif could be important for substrate positioning and the catalytic properties of 13R-MnLOX. We also considered the nearby Gly$^{335}$ of the Gly/Ala determinant of regio- and stereospecific (R/S) oxidation at C-1 and C-5 of the 1Z,4Z-pentadiene (34). A few LOXs, including 9S-MnLOX, deviate from this rule of R/S stereospecificity (35, 36). The Gly/Ala position also influences oxygen access and the hydroperoxide isomerase activities of eLOX-3 and 13R-MnLOX (37, 38).

13R-MnLOX is secreted by the take-all fungus and can be expressed in *Pichia pastoris* as a secreted, glycosylated protein of 602 amino acids (≈91 kDa; Refs. 11 and 28). Recombinant 13R-MnLOX is transformed by spontaneous hydrolysis during storage to a smaller enzyme of uniform size (≈67.4 kDa) with virtually unchanged catalytic activity (11). This mini 13R-MnLOX is formed by loss of a sequence of glycosylated amino acid residues at the N-terminal end of the C2 domain, but the cleavage position is unknown. A crystal screening of mini 13R-MnLOX suggested that this protein might be amenable for crystallization. This led us to investigate whether it could be possible to determine the cleavage position and to express recombinant mini 13R-MnLOX as mini 13R-LOX retained the catalytic parameters of the native enzyme (11) and to use it for further studies. A recent report demonstrates that the C2 domain of mammalian LOX has little influence on catalytic activity and regiospecificity (39).

The main objective of the present investigation was to study replacements near the catalytic metal of 13R-MnLOX based on crystal structural information on the substrate channels of sLOX-1, coral 8R-LOX, human 5-LOX, and rabbit 15-LOX covering an important region for regio- and stereospecificity. A, overview of hydrogen abstraction and oxygenation of 18:2n-6. Lipoxidation by sLOX-1 occurs by abstraction of the pro-S hydrogen at C-11 with antarafacial oxygen insertion at C-13 with formation of 13S-HPODE, whereas 9S- and 13R-MnLOX abstract the very same hydrogen but form 9S- and 13R-HPODE, respectively, as the main products (suprafacial oxygen insertion). B, partial amino acid sequences with the conserved determinant (Gly/Ala) for regio- and R/S stereospecificity and the Sloane determinant of regiospecificity (see Refs. 15, 16, 20, and 33) as marked by arrows. The catalytic importance of Leu$^{336}$ and Phe$^{337}$ of 13R-MnLOX was investigated in this report.

![FIGURE 1. Overview of possible lipoygenation positions of linoleic acid and partial alignment of 13R-MnLOX, sLOX-1, 8R-LOX, human 5-LOX, and rabbit 15-LOX covering an important region for regio- and stereospecificity.](image)

| Position | sLOX-1 | 13R-MnLOX | 8R-LOX | 5R-LOX | 15R-LOX |
|----------|--------|-----------|--------|--------|---------|
| 2         |        |           |        |        |         |
| 3         |        |           |        |        |         |
| 4         |        |           |        |        |         |
| 5         |        |           |        |        |         |
| 6         |        |           |        |        |         |
| 7         |        |           |        |        |         |
| 8         |        |           |        |        |         |
| 9         |        |           |        |        |         |
| 10        |        |           |        |        |         |
| 11        |        |           |        |        |         |
| 12        |        |           |        |        |         |
| 13        |        |           |        |        |         |
| 14        |        |           |        |        |         |
| 15        |        |           |        |        |         |
| 16        |        |           |        |        |         |
| 17        |        |           |        |        |         |
| 18        |        |           |        |        |         |
| 19        |        |           |        |        |         |
| 20        |        |           |        |        |         |
| 21        |        |           |        |        |         |
| 22        |        |           |        |        |         |
| 23        |        |           |        |        |         |
| 24        |        |           |        |        |         |
| 25        |        |           |        |        |         |
| 26        |        |           |        |        |         |
| 27        |        |           |        |        |         |
| 28        |        |           |        |        |         |
| 29        |        |           |        |        |         |
| 30        |        |           |        |        |         |
| 31        |        |           |        |        |         |
| 32        |        |           |        |        |         |
| 33        |        |           |        |        |         |
| 34        |        |           |        |        |         |
| 35        |        |           |        |        |         |
| 36        |        |           |        |        |         |
| 37        |        |           |        |        |         |
| 38        |        |           |        |        |         |
| 39        |        |           |        |        |         |
| 40        |        |           |        |        |         |
| 41        |        |           |        |        |         |
| 42        |        |           |        |        |         |

**EXPERIMENTAL PROCEDURES**

**Materials**—18:1n-6 (99%) was from Lipidox, 18:2n-6 (99%) was from Sigma, and 18:3n-3 (99%) was from Merck. Fatty acids were dissolved in ethanol and stored in stock solutions (30–100 mm) at −20 °C. Fresh solutions (50–100 μl) were prepared in 0.1 M NaBO$_3$ buffer (pH 9.0). pPICZaA, *P. pastoris* (strain X-33), and phleomycin (Zeocin) were from Invitrogen. Yeast nitrogen base was from Sigma and Invitrogen. DNA polymerases were from Fermentas (Pfu DNA polymerase) and Finzymes (Phire DNA polymerase). Oligonucleotides for PCR were obtained from TIB Molbiol (Berlin, Germany) and Cybergene (Huddinge, Sweden). Restriction enzymes were from New England Biolabs, Fermentas, and New England Biolabs.

$^{3}$ A. Wennman and E. H. Oliw, unpublished observations.
England Biolabs and Fermentas. Chemically competent *Escherichia coli* (NEB5α) were from New England Biolabs. Anion exchange columns for plasmid DNA purification (NucleoBond AX) were from Macherey-Nagel. Recombinant 13R-MnLOX_602 G332A was expressed as described (this Gly residue is number 316 of the mature sequence and number 332 of the precursor sequence; Ref. 38). 11R-HPOTrE and [11S,13H]18:2n-6 (99% ²H) were prepared as described (40, 41). Media and columns for protein purification were from GE Healthcare. Equipment and reagents for SDS-PAGE were from Bio-Rad. Sep-Pak C₁₈ cartridges were from Waters (Milford, MA).

**Expression Constructs of Mini 13R-MnLOX**—The 13R-MnLOX precursor consists of 618 amino acids (supplemental Fig. S1). 13R-MnLOX (602 amino acids) was previously expressed and secreted by *P. pastoris* using pPICZₐMnLOX_602 (11). This plasmid was used to obtain shorter expression constructs. The expression plasmid was modified by PCR technology to include an N-terminal His tag. In short, the codons for His₆-Gln-Gln-Leu were introduced by using the EcoRI restriction site in the cloning region of pPICZₐMnLOX (aatttcCATCATCATCATCATCTGcag) along with a restriction site for PstI (underlined). Next we created a unique restriction site for AflII (cttaag) without changing the coded sequence (L176K; numbered from the 13R-MnLOX precursor) by site-directed mutagenesis. This vector was restricted with PstI and AflII and then ligated with PCR fragments of the desired length to shorten the N-terminal end of the open reading frame. This yielded three constructs with 580, 570, or 555 residues to the C-terminal end (supplemental Fig. S1). This yielded three constructs with 580, 570, or 555 residues to the C-terminal end (supplemental Fig. S1). pPICZₐ-Mini-MnLOX₅₈₀ thus coded for the sequence Glu-His₆-Gln-Gln-Leu-Thr₁₁₋₁₅₋₆₁₈. The DNA sequence differed in two positions (K₅₂N and Y₁₅₈C) from the sequence reported in GenBank™ (accession number AAK81882).

**Site-directed Mutagenesis of 13R-MnLOX**—pPICZₐ-MnLOX₅₈₀ was modified by replacements of Leu₃₃₆ and Phe₃₃₇ by site-directed mutagenesis using *Pfu* and Phire polymerases and the oligonucleotides listed in supplemental Table S1. For L336A and L336G, the pPICZₐMnLOX₅₈₀_L336V was expressed as described (this Gly residue is numbered from the 13R-MnLOX precursor) by site-directed mutagenesis. This vector was restricted with PstI and AflII and then ligated with PCR fragments of different length to shorten the N-terminal end of the open reading frame. This yielded three constructs with 580, 570, or 555 residues to the C-terminal end (supplemental Fig. S1). pPICZₐ-Mini-MnLOX₅₈₀ thus coded for the sequence Glu-His₆-Gln-Gln-Leu-Thr₁₁₋₁₅₋₆₁₈. The DNA sequence differed in two positions (K₅₂N and Y₁₅₈C) from the sequence reported in GenBank™ (accession number AAK81882).

**Expression of Recombinant Enzymes**—*P. pastoris* was transformed as described (11). Transformants were selected on yeast peptone-dextrose-agar plates with Zeocin (100 μg/ml) at 28 °C (11). PCR analysis of genomic DNA was used to confirm recombination of the plasmid into the genomic DNA (42). Resistant colonies were first grown to generate biomass and then grown in buffered minimal medium described (11). Protein biosynthesis was induced by 0.5–1% methanol added daily in the medium for 4–5 days (11, 43). The *Pichia* cells were harvested by centrifugation (5,000 × g) and could be reused for additional expression. Secreted recombinant 13R-MnLOX was purified from the supernatant after centrifugation of the yeast suspension.

**Enzyme Purification**—Solid (NH₄)₂SO₄ was added to the supernatant to a concentration of ~1 M, and the pH was adjusted to 7.0–7.2 with 10 M KOH. After centrifugation and filtration, the material was loaded on a column for hydrophobic interaction chromatography (33 × 40 mm, phenyl-Sepharose) in 1 M (NH₄)₂SO₄, 0.025 M potassium phosphate buffer (pH 7.1) and washed with the same buffer, and absorbed proteins were eluted with 25 mM potassium phosphate buffer (pH 7.1) (28). The column was washed with 70% ethanol and 6 M urea before the next analysis. The peak fractions were combined, concentrated to 1–2 ml by diafiltration (Ultracel 30K, Millipore), and stored at 4 °C in 0.5–2 ml of 100 mM Tris-HCl (pH 8), 150 mM NaCl, 0.04% Tween 20, 1 mM NaN₃. The proteins were analyzed by SDS-PAGE, and 13R-MnLOX and its mutants constituted at least 25–30% of the proteins as judged from the SDS-PAGE analysis (see Fig. 2A). Further partial purification was achieved by gel filtration (Superdex 200 HR 10/30) in 0.05 M potassium.
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phosphate buffer (pH 7.4), 0.15 M NaCl, 1 mM NaN₃, 0.04% Tween 20 with UV detection (280 nm).

Lipoxygenase Assay and UV 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⁻¹ M⁻¹. LOX activity was monitored by UV spectroscopy (237 nm) in 0.1 M NaBO₃ (pH 9.0) with 50–100 μM 18:3n-3 and 18:2n-6 as substrates. Apparent Km values were estimated with 18:3n-3 (2.5–55 μM) in triplicates from the linear rate of biosynthesis of cis-trans conjugated products. We used GraFit (Erithacus Software) for non-linear estimates of Km values, based on seven to eight data points, which were fitted to the Michaelis-Menten equation. Specific activities were determined with 100 μM 18:3n-3.

Oxidation of 18:1n-6 was performed with 13R-MnLOX as described (30). The enzymatic products were analyzed by LC-MS after extractive isolation (SepPak C₁₈). Protein concentration was estimated by UV absorption at 280 nm (NanoDrop ND-1000 spectrophotometer).

13R-MnLOX forms 11S-HPODE and 11R-HPOTrE as intermediates during the linear phase of oxidation and the 13R-hydroperoxides as main end products (26). The products formed by the mutants were therefore analyzed at two time points: when the UV absorbance at 235 or 237 nm had ceased to increase (apparent end point; UV absorption, 1.5–2 absorbance units) and at the middle of the linear increase in UV absorbance (midpoint; UV absorption, 0.5–1 absorbance unit).

LC-MS/MS Analysis—Reversed phase HPLC was performed with a Surveyor MS pump (ThermoFisher) and an octadecyl (midpoint; UV absorption, 0.5–1 absorbance unit). The heated transfer capillary was set at 315 °C, the isolation width was set at 1.5 or 5 (for unstable hydroperoxides; Refs. 40). The heated transfer capillary was set at 315 °C, the isolation width was set at 1.5 or 5 (for unstable hydroperoxides; Refs. 40). The heated transfer capillary was set at 315 °C, the isolation width was set at 1.5 or 5 (for unstable hydroperoxides; Refs. 40).

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LC-MS/MS Analysis—Reversed phase HPLC was performed with a Surveyor MS pump (ThermoFisher) and an octadecyl silica column (5 μm; 2 × 150 mm; Phenomenex; equipped with 2 × 4-mm C₁₈ guard cartridge), which was eluted at 0.3 ml/min with 750:250:0.1 or 800:200:0.1 methanol/water/acetic acid. The effluent was subject to electrospray ionization in a linear ion trap mass spectrometer (LTQ, ThermoFisher) as described (40). The heated transfer capillary was set at 315 °C, the isolation width was set at 1.5 or 5 (for unstable hydroperoxides; Refs. 44 and 45), and the collision energy was set at 35%. A trihydroxy fatty acid (prostaglandin F₁₂₀, 10 ng/min) was infused for tuning. Hydroperoxides were occasionally reduced to alcohols by treatment with triphenylphosphine (1–10 μg) before LC-MS/MS analysis. Normal phase HPLC was performed as described (30).

Chiral Phase HPLC-MS analysis of hydroxy fatty acids was performed with Reprosil Chiral-AM (amylose tris(3,5-dimethylphenylcarbamate)-modified silica; 2 × 250 mm; equipped with a 2 × 4-mm silica cartridge) eluted at 0.2 ml/min (with either 95:5:0.01 hexane/methanol/acetic acid or 93:5:2:0.01 hexane/methanol/ethanol/acetic acid; Ref. 46) or with Chiralcel OBH (Daicel; 4.6 × 250 mm; equipped with a silica guard cartridge) eluted with 95:5:0.1 hexane/isopropyl alcohol/acetic acid at 0.5 ml/min. Hydroperoxy fatty acids were separated on Reprosil Chiral NR (8 μm; 2 × 250 mm; Dr. Maisch GmbH, Ammerbuch, Germany; equipped with a silica guard cartridge) eluted at 0.5 ml/min with 99:1:0.1 hexane/methanol/acetic acid (45). The effluent from the chiral columns was combined with 3:2 isopropl alcohol/water from a Surveyor MS pump (0.1–0.25 ml/min) and analyzed on line by MS/MS as described above.

Miscellaneous Methods—SDS-PAGE was performed as described (11). Protein bands were excised and treated with trypsin as described (47). Peptides were analyzed by MALDI-TOF (Bruker Ultraflex TOF/TOF), and the Mascot program was used for analysis. N-terminal sequencing of mini 13R-MnLOX was performed at the Protein Analysis Center, Karolinska Institute, Stockholm, Sweden.

RESULTS

Sequencing and Expression of Mini 13R-MnLOX—Secreted 13R-MnLOX of 602 amino acids was transformed during storage to a smaller and less glycosylated protein of ~67 kDa that retained the enzyme activity and the catalytic parameters of native 13R-MnLOX (11).

SDS-PAGE purification, trypsin digestion, and MALDI-TOF analysis of peptides showed that this mini form of 13R-MnLOX contained at least 562 C-terminal amino acids (supplemental Fig. S1). Sequential N-terminal sequencing suggested that mini 13R-MnLOX was mainly formed by cleavage between Thr37 and Thr38 as judged from five amino acids in correct sequence order from this point (data not shown). This corresponded to a protein of 580 amino acids with a calculated molecular mass of 63.8 kDa.

We expressed 13R-MnLOX with 580, 570, and 555 amino acids and confirmed protein expression by SDS-PAGE. The 580-amino acid construct retained catalytic activity, whereas the two shorter constructs were inactive. We confirmed the structure by MALDI-TOF analysis of tryptic peptide fragments.

The recombinant mini 13R-MnLOX of 580 residues oxidized 18:2n-6 with formation of 11- and 13-hydroperoxy metabolites as formed by native 13R-MnLOX. Unexpectedly, recombinant mini 13R-MnLOX was glycosylated and in some experiments to the same extent as recombinant 13R-MnLOX_602; SDS-PAGE thus revealed a broad protein band due to heterogenous glycosylation (Fig. 2A). The recombinant mini form with 580 amino acids was used in this report unless otherwise stated as its catalytic activities appeared to be identical to those of 13R-MnLOX (see Ref. 11), and this recombinant minienzyme is referred to as 13R-MnLOX for simplicity.

The effects of replacements at the Leu336 and Phe337 positions of 13R-MnLOX on catalysis are summarized in Tables 1 and 2. Except for L336V, the catalytic efficiency of the mutants was markedly reduced.

Replacement of Phe337 of 13R-MnLOX with Ile, Val, and Ala—Replacement of Phe337 with Ile mainly changed the steric oxidation of 18:2n-6 and 18:3n-3 at C-13 but also increased oxygenation at C-9 (Table 1).

The F337I mutant of 13R-MnLOX (F337I_13R-MnLOX) oxidized 18:2n-6 to 13S- and 13R-HPOTrE in a ratio of 3:1 as the major end products, and 18:2n-6 was oxidized in nearly the same way (Table 1 and Fig. 2, B and C). The oxidation at C-9 was enhanced compared with 13R-MnLOX, and the stereospecificity was altered. 18:2n-6 was transformed mainly to the 9S-hydroperoxy stereoisomer, and 18:3n-3 was transformed mainly to the 9R-hydroperoxy stereoisomer (Table 1). F337V_13R-MnLOX and F337A_13R-MnLOX were also expressed as...
TABLE 1
Overview of the regio- and stereospecific oxygenation of 18:2n-6 and 18:3n-3 by 13R-MnLOX and mutants and their apparent catalytic activities

| Regiospecificity (stereospecificity) and specific activities* | 18:2n-6 | 18:3n-3 |
|---------------------------------------------------------------|--------|--------|
| Enzyme, Footnote b)                                          | C-13 (R) | C-9 (S) | C-13 (R) | C-9 (S) | Specific activity |
| L336V                                                        | 97 (>99) | 3 (>90) | 97 (>99) | 2–3 (>95) | 100 |
| L336A                                                       | 89 (>99) | 11 (70) | 94 (>99) | 6 (17)  | ~48 |
| L336G                                                       | 39 (>98) | 61 (44) | 34 (88)  | 66 (17) | <0.2 |
| F337I                                                       | 74 (>98) | 22 (19) | 78 (>98) | 22 (11) | <0.2 |
| L336F                                                       | 86 (>98) | 10 (57) | 90 (>98) | 10 (22) | ~1.5 |
| F337I                                                       | 77 (31)  | 23 (78) | 94 (26)  | 6 (33)  | ~0.7 |

* Relative data from analysis of end products from incubation with 13R-MnLOX and its mutants with 100 μM substrate in 0.1 M NaBO3 (pH 9.0). The relative amounts of products were estimated by selective ion chromatograms and gel filtration, and these numbers provide an index of catalytic turnover. L336V designates recombinant 13R-MnLOX.

These enzymes oxidized [11S-2H]18:2n-6 with a kinetic isotope effect (kH/kD) of ~32 (L336V) and ~23 (L336A; see Ref. 33) and with apparent loss of the deuterium label of 13-HODE as judged from LC-MS analysis. The kH/kD of L336A was not further investigated due to low specific activity.

TABLE 2
Apparent Km values for oxidation of α-linolenic acid to cis-trans conjugated hydroperoxides with UV absorbance at 237 nm by 13R-MnLOX and its Leu336 and Phe337 mutants

| Enzyme | L336V | L336A | L336G | L336F | F337I |
|--------|-------|-------|-------|-------|-------|
| Km (μM) | 0.5 ± 0.1 | 2.7 ± 0.4 | 3.6 ± 0.8 | 6.5 ± 1.0 | 6.5 ± 0.9 | 7.3 ± 1.0 |

* Mean and S.D. of Km values (μM) were deduced from non-linear analysis of the Michaelis-Menken equation by GraFit software. L336V designates recombinant 13R-MnLOX.

FIGURE 3. Chiral phase HPLC-MS/MS analysis of products formed by the L336G-13R-MnLOX and L336F-13R-MnLOX. A, oxidation of 18:2n-6 by L336G-13R-MnLOX. Peaks in l–lll were identified as indicated by the selective ion chromatograms (MS/MS analysis, m/z 295 → full scan) of 13- and 9-HODE. A small amount of racemic [13C10]-9- and 9-HODE was added to facilitate identification of stereoisomers. B, oxidation of 18:3n-3 by L336F-13R-MnLOX. The metabolites in peaks I–IV were identified by selective ion chromatograms as shown and by complete MS spectra. Top, total ion current (TIC). Shown below are characteristic ion chromatograms for 13-HOTrE, 9-HOTrE, and 11-HOTrE; the stereoisomers of 11-HOTrE were not resolved (Chiralcel OB).

L336A-13R-MnLOX and L336G-13R-MnLOX showed low but detectable catalytic activities (less than 0.2% of 13R-MnLOX; Table 1). L336A-13R-MnLOX increased the oxidation at C-9 of 18:2n-6 so that 13- and 9-HPODE were formed in a ratio of ~2:3. Only small amounts of 11-HPODE were detected during the linear phase of oxidation (data not shown). 13R-HPODE was formed with high stereoselectivity, whereas 9-HPODE was almost racemic. L336A oxidized 18:3n-3 to 9R-HPOTrE as the main metabolite (Table 1). L336G-13R-MnLOX formed 13R-HPODE and 13R-HPOTrE as the main products (73–74%) but yielded 9-hydroperoxides predominantly with R configuration (Fig. 3 and Table 1).

Replacement of Leu336 and Gly332 with Larger Hydrophobic Residues—Replacement of Leu336 with Phe had relatively small effects on the regiospecific oxygenation of 18:2n-6 and 18:3n-3 as 13R-hydroperoxy metabolites remained the main products (86–90%; Table 1). This is also in analogy with G332A, which only slightly increased the oxygenation at C-9 (38). Km for 18:3n-3 remained in the low μM range for L336F (Table 2). For comparison, Km for G332A-13R-MnLOX was determined to be 2.5 ± 0.8 μM.

L336F-13R-MnLOX oxidized 18:2n-6 to products with UV absorbance at 235 nm as shown in comparison with L336V-13R-MnLOX in Fig. 4A. After a prolonged kinetic lag phase, the absorption reached a maximum and then began to decline. This phenomenon was augmented with 18:3n-3 as a substrate (Fig. 4B). As the absorbance at 237 nm declined, there was an increase in UV absorbance at 280 nm (Fig. 4B, inset) apparently due to dehydration of 13R-HOTrE. These events were unchanged in buffer saturated with oxygen (Fig. 4B); the lag phase appeared to be shortened in oxygen-saturated buffer, but this was not further investigated.

G332A-13R-MnLOX has previously been found to enhance the dehydration of 13R-HOTrE (38). A notable difference...
DISCUSSION

We report two main observations. First, F337I-13R-MnLOX changed the absolute configuration of the main 13-hydroperoxy metabolites of 18:2n-6 and 18:3n-3 from ~100% R to ~70% S but did not alter abstraction of the pro-S hydrogen at C-11 of 18:2n-6. The hydrogen abstraction and oxygenation was thus altered from exclusively suprafacial to predominantly antarafacial, which mimics the antarafacial oxygenation mechanism of sLOX-1 and other FeLOXs. Second, replacement of Leu$_{336}$ with smaller or larger hydrophobic residues changed the region and stereospecific oxygenation of C$_{18}$ fatty acids in a substrate-dependent way, the disposition of molecular oxygen to the catalytic center, and the hydroperoxide isomerase activity. An overview of these effects is shown in Fig. 6. All mutants except L336V showed markedly reduced specific activities (Table 1).

Site-directed mutagenesis of the active site of LOX can change the oxygen insertion in three principal ways, whereas the hydrogen abstraction remains invariant. First, replacements with small or large hydrophobic residues can shift the position of hydrogen abstraction at C-3 and oxygenation at C-5 of one 1Z,4Z-pentadiene of the substrate to these positions of the overlapping, vicinal 1Z,4Z-pentadiene as first described for 15-LOX by Sloane et al. (20). This finding was recently extended to a triad model for substrate positioning (48). By a similar mechanism, swapping Gly and Ala at one specific position of many LOXs changed the stereochemistry of the main product by oxygen insertion at the opposite ends of the 1Z,4Z-pentadiene (34). The substrate alignment in the active site is changed, but antarafacial hydrogen abstraction and oxygen insertion are retained (34). Second, the substrate can bind to the active site in different orientations. At acidic pH, the uncharged carboxyl group of 18:2n-6 can enter the active site of sLOX-1 for oxidation in reverse head-tail orientation (49). In analogy,
replacement of a hydrophobic amino acid at the bottom of the substrate channel of cucumber 13-LOX unmasked a positively charged residue and shifted oxygenation from C-13 to C-9 (50). This mechanism was recently highlighted by conversion of human 5-LOX to 15-LOX by replacement of Ser^{663} with an Asp residue, a mimic of phosphorylation (18). The antarafacial relationship between hydrogen abstraction and oxygenation is not altered by the substrate in the opposite head-tail orientation. Third, radical leakage may be increased by structural perturbations of the active site in analogy with the radical leakage of sLOX-3. The latter releases the 1\textsuperscript{Z},4\textsuperscript{Z}-pentadiene radical so that an almost racemic mixture of hydroperoxides is generated.

**Catalytic Convergence**—F337I:13R-MnLOX describes an unprecedented modification of the oxidation process. 13R-MnLOX and its 9S-MnLOX homologue of *M. salvinii* are characterized by suprafacial hydrogen abstraction and oxygen insertion (26).\(^3\) The F337I mutant retained this hydroxy abstraction but changed oxygen insertion from suprafacial to mainly antarafacial. Binding of 18:2\textsuperscript{n-6} and 18:3\textsuperscript{n-3} in the reverse orientation would be expected to give 9R-hydroperoxy metabolites as the main products, but this was not the case. The F337I mutant likely altered the oxygenation at C-13 from R to S by allowing oxygen access to the other face of the 12Z double bond. A comparison of the active sites of 13R-MnLOX and sLOX-1 based on modeling is shown in Fig. 7. It seems likely that Phe\textsuperscript{337} of 13R-MnLOX shields the face of the 12Z double bond, which is subject to 13S oxygenation by sLOX-1, whereas Ile\textsuperscript{347} of sLOX-1 shields this double bond for oxygen insertion at C-13 with R configuration. Schematic views of these steric effects of Phe\textsuperscript{337} and Ile\textsuperscript{347} are presented in Fig. 7, C and D.

It will be of interest to determine whether replacement of Ile or Val at this position could influence the steric oxidation of other LOXs in analogy with 13R-MnLOX. 8R-LOX contains an Ile residue in this position that has been replaced by Trp and Ala residues. I433A:8R-LOX retained catalytic activity and slightly changed the stereospecific oxidation ratios in the direction of suprafacial hydrogen abstraction and oxygen insertion (8R/8S, 95:5; 12R/12S, 16:84), whereas I433W:8R-LOX was inactive (16).

Unshielding of the 9Z Double Bond for Oxygen Insertion—We evaluated the effects of substitutions of Leu\textsuperscript{336} in 13R-MnLOX with 18:3\textsuperscript{n-3} and 18:2\textsuperscript{n-6} as substrates. This Leu residue is conserved in all LOXs and assumed to position the substrate near the metal for oxygenation (16, 33). 18:2\textsuperscript{n-6} and 18:3\textsuperscript{n-3} were oxidized by all Leu\textsuperscript{326} mutants at C-13 as the major products.
Control of Stereospecific Oxygenation by 13R-MnLOX

(≥74%) and consistently with >98% R configuration with only one exception, L336A (Table 1). Mutations at the Leu336 position seemed to influence the oxygenation at C-9. The absolute configuration at C-9 was mainly 9R with 18:3n-3 as a substrate. These changes from 9S to 9R configuration were less pronounced with 18:2n-6. Substituting Leu with Val thus had smaller effects on 18:2n-6 (70% 9R) than on 18:3n-3 (17% 9R). These results support that Leu336 restrains the substrate for stereospecific oxygenation insertion in the 9S position, which can be perturbed by replacements.

The replacements of 13R-MnLOX at the Leu336 position had little influence on $K_m$ for 18:3n-3 in comparison with the markedly reduced lipoxygenation (Tables 1 and 2). For comparison of homologous replacements, L546A sLOX-1 lost over 98% of total catalytic activity and increased the relative oxygenation at C-9 of 18:2n-6 to 13%. The latter was attributed to reverse substrate orientation in the active site (33). The L432A and L432F mutants of 8R-LOX also lost most of the enzyme activity (≥95%), but they increased the oxygenation of 20:4n-6 at C-12 from 2 to 10% and to 34%, respectively, with retention of the R configuration at C-8; L432V and L432I did not affect the regio- and stereospecificity (16). We conclude that hydrophobic replacements of Leu336 changed the relative oxygenation at C-13 and C-9 of 18:2n-6 and 18:3n-3 in analogy with L546A sLOX and in analogy with oxidation of 20:4n-6 by mutants of coral 8R-LOX.

Oxygen Access—As previously reported, G332A:13R-MnLOX augmented the hydroperoxide isomerase activity and the formation of epoxy alcohols from 13R-HPOTrE (38). We found that L336F:13R-MnLOX also possessed prominent hydroperoxide isomerase activity. In contrast to G332A:13R-MnLOX, the hydroperoxide isomerase activity of L336F:13R-MnLOX could not be reduced in oxygen-saturated buffer. The decrease of the catalytic space with G332A or L336F replacement likely restricts the disposition of molecular oxygen in the active site. Interestingly, Zheng and Brash (51) found that A451G eLOX-3 allowed molecular oxygen access to the enzyme center, which reduced the hydroperoxide isomerase activity and revealed LOX activity.

Summary—We report that F337I:13R-MnLOX changed the stereospecificity and formed the 13S-hydroperoxides of 18:2n-6 and 18:3n-3 as main metabolites. This occurred by antarafacial hydrogen abstraction and oxygenation in analogy with sLOX-1. The Leu336 residue was important for substrate-dependent regio- and stereospecific oxygenation at C-9, oxygen access to the catalytic center, and consequently the hydroperoxide isomerase activity (51).

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