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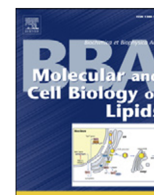

Manganese lipoxygenase oxidizes *bis*-allylic hydroperoxides and octadecenoic acids by different mechanisms

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ABSTRACT

Manganese lipoxygenase (MnLOX) oxidizes (11*R*)-hydroperoxylinolenic acid (11*R*-HpOTrE) to a peroxyl radical. Our aim was to compare the enzymatic oxidation of 11*R*-HpOTrE and octadecenoic acids with LOO—H and allylic C—H bond dissociation enthalpies of ~88 and ~87 kcal/mol. Mn(III)LOX oxidized (11*Z*)-, (12*Z*)-, and (13*Z*)-18:1 to hydroperoxides with *R* configuration, but this occurred at insignificant rates (<1%) compared to 11*R*-HpOTrE. We next examined whether transitional metals could mimic this oxidation. Ce^{4+} and Mn^{3+} transformed 11*R*-HpOTrE to hydroperoxides at C-9 and C-13 via oxidation to a peroxyl radical at C-11, whereas Fe^{3+} was a poor catalyst. Our results suggest that MnLOX oxidizes *bis*-allylic hydroperoxides to peroxyl radicals in analogy with Ce^{4+} and Mn^{3+}. The enzymatic oxidation likely occurs by proton-coupled electron transfer of the electron from the hydroperoxide anion to Mn(III) and H^+ to the catalytic base, Mn(III) OH^-. Hydroperoxides abolish the kinetic lag times of MnLOX and FeLOX by oxidation of their metal centers, but 11*R*-HpOTrE was isomerized by MnLOX to (13*R*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13*R*-HpOTrE) with a kinetic lag time. This lag time could be explained by two competing transformations, dehydration of 11*R*-HpOTrE to 11-ketolinolenic acid and oxidation of 11*R*-HpOTrE to peroxyl radical; the reaction rate then increases as 13*R*-HpOTrE oxidizes MnLOX with subsequent formation of two epoxyalcohols. We conclude that oxidation of octadecenoic acids and *bis*-allylic hydroperoxides occurs by different mechanisms, which likely reflect the nature of the hydrogen bonds, steric factors, and the redox potential of the Mn(III) center.

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1. Introduction

Lipoxygenases occur in plants, mammals, and a few microorganisms and are of considerable medical, biological, and mechanistic interest [1–3]. These non-heme metalloenzymes oxygenate polyunsaturated fatty acids to hydroperoxides and can convert hydroperoxides to allylic epoxides, leukotrienes, and epoxyalcohols (1–3). The prototypes are soybean lipoxygenase-1 (sLOX-1), arachidonate 5-LOX, and epidermal LOX-3 [4], respectively. All lipoxygenases belong to the same gene family, and the catalytic metal is iron with only one known exception, the manganese lipoxygenase (MnLOX) of the Take-all fungus of wheat [5]. The catalytic metal (M) is ligated to water, and the metal redox cycles during catalysis between reduced, inactive ($M^{2+}OH_2$) and oxidized, active ($M^{3+}OH^-$) enzyme states.

MnLOX is so far the only characterized fungal LOX, and it belongs to a subfamily with a C-terminal valine residue as a characteristic feature, whereas isoleucine is usually found at this position of mammalian and plant LOX. MnLOX also has a unique reaction mechanism. It oxidizes α-linolenic to ~80% 13*R*-HpOTrE, ~20% 11*R*-HpOTrE, and to traces of 9*S*-HpOTrE [5,6]. MnLOX forms 11*R*-HpOTrE and 13*R*-HpOTrE by suprafacial hydrogen abstraction (Fig. 1A) and oxygen insertion, whereas all other LOX catalyze antarafacial hydrogen abstractions and oxygenations. MnLOX isomerizes 11*R*-HpOTrE to 13*R*-HpOTrE, and this occurs with reformation of the peroxyl radical at C-11. The latter will rapidly undergo β-fragmentation with a rate constant of $\sim 2 \times 10^6$ [7,8]. Studies with $^{18}O_2$-labeled 11*S*-HpODE confirmed that 13*R*-HpODE retained the $^{18}O_2$-label in agreement with this mechanism [6]. A note on the nomenclature is warranted, as linoleic acid and α-linolenic acid are oxygenated at C-11 to identical *bis*-allylic hydroperoxides, which will be labeled *S* and *R* due to the absence or presence of an n-3 double bond. *bis*-Allylic hydroperoxides are also formed by coral 8*R*-LOX and by a recently

Abbreviations: BDE, bond dissociation enthalpy; CP, chiral phase; HOME(m*Z*), hydroxy-(m*Z*)-octadecenoic acid; HOTrE, hydroxyoctadecatrienoic acid; HPLC, high performance liquid chromatography; HpODE, hydroperoxyoctadecadienoic acid; HpOME, hydroperoxyoctadecenoic acid; HpOTrE, hydroperoxyoctadecatrienoic acid; KOME, ketooctadecenoic acid; KOTrE, keto-octadecatrienoic acid; LC, liquid chromatography; LOX, lipoxygenase; MnLOX, manganese lipoxygenase; MS, mass spectrometry; (m*Z*)-18:1, (m*Z*)-octadecenoic acid; 4-NC, 4-nitrocatechol; MS/MS, tandem MS; NL, normalized; NP, normal phase; PCET, proton-coupled electron transfer; PGH, prostaglandin H; RP, reverse phase; sLOX-1, soybean lipoxygenase-1; TIC, total ion current

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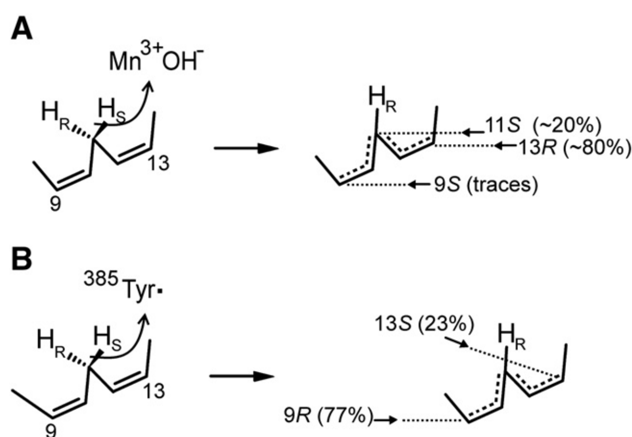

Fig. 1. Schematic overview of the mechanism of hydrogen abstraction by MnLOX and PGH synthase-1. A, Abstraction of the pro-*S* hydrogen at C-11 of (9*Z*,12*Z*)-18:2 by the catalytic base of MnLOX. B, Abstraction of the pro-*S* hydrogen at C-11 of (9*Z*,12*Z*)-18:2 by the tyrosyl radical of PGH synthase-1 [34]. The direction of oxygenation is marked by arrows (suprafacial hydrogen abstraction and oxygenation in A, antarafacial in B). Due to the nomenclature rules, the 11-hydroperoxide formed in A from (9*Z*,12*Z*)-18:2 will be designated 11*S*, whereas the corresponding hydroperoxides of (9*Z*,12*Z*,15*Z*)-18:3 will be designated 11*R* due to the n-3 double bond.

described mini-LOX of *Cyanothece* SP [9,10], and they are therefore not specific for MnLOX.

Little is known about the enzymatic oxidation of hydroperoxides to peroxyl radicals, but the non-enzymatic formation of peroxyl radicals during autoxidation of fatty acids has been well characterized. The LOO—H bond dissociation enthalpy (BDE) is ~88 kcal/mol [11], whereas the C—H BDE of the *bis*-allylic hydrogen of linoleic acid and the allylic hydrogens of 18:1 are ~73 kcal/mol and ~87 kcal/mol, respectively [11]. Polyunsaturated fatty acids are thus more susceptible to autoxidation than octadecenoic acids. Peroxyl radicals can be formed from hydroperoxides by strong oxidants (e.g., alkoxyl radicals, transitional metals, and HOCl), and the redox potentials of a series of biochemical half reactions are listed in Table 1. Peroxyl radicals are directly formed during autoxidation of polyunsaturated fatty acids by oxygenation of carbon-centered pentadienyl radicals, and they can propagate autoxidation by hydrogen abstraction of *bis*-allylic carbons (cf. Table 1).

How does the metal center of MnLOX oxidize *bis*-allylic hydroperoxides to peroxyl radicals? The mechanism could be either

Table 1
Biochemical half reactions of reactive oxygen species, metal ions, and oxidants.

Biochemical half reactions (pH 7)	E°′/V	Refs.
ROH↔RO· + e⁻ + H⁺	1.6	[43]
LOOH↔LOO· + e⁻ + H⁺	0.77–1.44	[43]
in alkylperoxide	1.0	[43]
$2H_2O \leftrightarrow O_2(aq) + 4e^- + 4H^+$	0.85 (0.73 at pH 9)	[44]
H-allyl↔allyl· + e⁻ + H⁺ in cyclopentene	0.70	[45]
PUFA↔PUFA· + e⁻ + H⁺ (polyunsaturated fatty acid, *bis*-allylic-H)	0.60	[43]
4-NC ↔ 4-NsQ⁻ + 2 H⁺ + e⁻	0.68 (0.57 at pH 9)	[31]
Fe(II)LOX ↔ Fe(III)LOX + e⁻	~0.6	[31]
HOCl[a] ↔ ·OCl + e⁻ + H⁺	0.170–0.250[a]	[46]
$Fe^{2+} \leftrightarrow Fe^{3+} + e^-$	0.11	[43]
Biochemical half reactions (pH 0)	**E°/V**	**Refs.**
$Ce^{3+} \leftrightarrow Ce^{4+} + e^-$	1.72	[47]
$Mn^{2+} \leftrightarrow Mn^{3+} + e^-$	1.54	[47]
$Fe^{2+} \leftrightarrow Fe^{3+} + e^-$	0.77	[47]
Fe(II)LOX ↔ Fe(III)LOX + e⁻	~1.1	[12]

[a] Hydroperoxides may be formed by HOCl from an unstable chlorinated peroxyl intermediate (LOOCl → LOO· + ·Cl) [46]. Abbreviations: sQ, semiquinone.

analogous to the enzymatic abstraction of the *bis*-allylic hydrogen of linoleic acid or different due to the chemical properties of the hydroperoxide group. Experimental and density-functional analyses of lipoxygenation suggest a proton-coupled electron transfer mechanism (PCET) by which the electron and the proton are transferred separately [12], the electron to the metal center and the proton to the catalytic base. Abstractions of *bis*-allylic hydrogens by lipoxygenases are associated with a large kinetic isotope effect (k_H/k_D ~40), which is due to tunneling [13]. In contrast, deuterium labeling of the 11-hydroperoxide had little effect on the rate of transformation of 11*S*-HpODE to 13*R*-HpODE [14]. This implied that the mechanism of proton transfer might differ between C—H and LOO—H oxidations.

The almost identical BDE of LOO—H and C—H of the allylic hydrogens of octadecenoic acids seemed worth investigating in relation to the catalytic properties of MnLOX for two reasons. First, a comparison of the oxidation efficiency of octadecenoic acids and *bis*-allylic hydroperoxy fatty acids by MnLOX might provide information on the catalytic mechanisms. Second, the importance of the metal centers for oxidation of octadecenoic acids and *bis*-allylic hydroperoxides might be deduced from a comparison of MnLOX and FeLOX. sLOX-1 oxidizes (12*Z*)-18:1 and (9*Z*)-18:1 slowly to enones [15,16], and transforms 11*S*-HpODE to 9- and 13-HpODE at low rates compared to MnLOX [10,17]. The main goals of the present report were therefore to study the mechanism of oxidation of 11*R*-HpOTrE to an 11-peroxyl radical and to determine whether MnLOX could oxidize a series of (m*Z*)-octadecenoic acids [(m*Z*)-18:1] to hydroperoxides. For steric analysis of these products, we prepared standards by oxidation of (11*Z*)-, (12*Z*)-, and (13*Z*)-18:1 with prostaglandin H (PGH) synthase-1, as this enzyme differs from MnLOX by catalyzing antarafacial hydrogen abstraction and oxygenation (Fig. 1B).

2. Materials and methods

2.1. Materials

(9*Z*,12*Z*,15*Z*)-18:3 (99%), HPLC solvents (Lichrosolve), manganese (III) acetate 2-hydrate (98%), $FeCl_3(H_2O)_6$, and most chemicals were from Merck. (9*Z*)-18:1 (99%) and (11*Z*)-18:1 (99%) were from Larodan, and (8*Z*)-18:1(99%) and (12*Z*)-18:1 (99%) were from Lipidox. (9*E*)-18:1 was from Applied Science Laboratories. 4-NC (97%), (9*Z*,12*Z*)-18:2 (99%), (13*Z*)-18:1 (99%), $(NH_4)_2Ce(NO_3)_6$, and sLOX-1 (4.4×10^5 units/mg; Lipoxidase) were from Sigma-Aldrich. Fatty acids were dissolved in ethanol and stored in stock solutions (50–100 mM) at −20 °C. Epoxyalcohols of 13*R*-HpOTrE were obtained and analyzed as described [18]. 12(13)Epoxy-(11*R*)-hydroxy-(9*Z*,15*Z*)-octadecadienoic acids were prepared by treatment of 11*R*-HOTrE with 1.5eq. m-chloroperoxybenzoic acid in CH_2Cl_2 [19]. 10*S*-HpOME(8*E*) was obtained as described [20]. 13-HpOME(14*E*) and 14-HpOME(12*E*) were obtained by photo oxidation of (13*Z*)-18:1 as described [20]. Recombinant MnLOX was expressed in *Pichia pastoris* (strain X-33) as a secreted and glycosylated protein of 604 amino acids (protein mass 67.7 kDa) using the expression vector pPICZA, and purified as described [5,21] with minor modifications (Supplemental data); SDS-PAGE analysis yielded one protein band, and the Mn content was in agreement with a mononuclear metal center [5,21]. Microsomes of ram seminal vesicles were prepared as described [22]. Nordihydroguaiaretic acid was provided by Dr. Hamberg (Karolinska Institutet).

2.2. Preparation of 11R-HpOTrE

11*R*-HpOTrE was isolated during the linear phase of oxidation of 100 µM (9*Z*,12*Z*,15*Z*)-18:3 by MnLOX [14]. After rapid extractive isolation on several cartridges (SepPak/C_{18}; Waters) in parallel, the products were separated by RP-HPLC [10 × 150 mm Reprosil 100 ODS-A; eluted at 2 ml/min (methanol/water/acetic acid, 800/200/0.05;

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