Kinetics of Manganese Lipoxygenase with a Catalytic Mononuclear Redox Center*

Received for publication, February 21, 2000, and in revised form, March 28, 2000
Published, JBC Papers in Press, April 5, 2000, DOI 10.1074/jbc.M001408200

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Manganese lipoxygenase was isolated from the take-all fungus, Geaeumannomyces graminis, and the oxygenation mechanism was investigated. A kinetic isotope effect, k_H/k_D = 21–24, was observed with [U-2H]linoleic acid as a substrate. The relative biosynthesis of (11S)-hydroperoxylinoiene (11S-HPODE) and (13R)-hydroperoxylinoiene (13R-HPODE) was pH-dependent and changed by [U-2H]linoleic acid. Stopped-flow kinetic traces of linoleic and α-linolenic acids indicated catalytic lag times of ∼45 ms, which were followed by bursts of enzyme activity for ∼60 ms and then by steady state (k_cat = 26 and 47 s⁻¹, respectively). 11S-HPODE was isomerized by manganese lipoxygenase to 13R-HPODE and formed from linoleic acid at the same rates (k_cat = 7–9 s⁻¹). Catalysis was accompanied by collisional quenching of the long wavelength fluorescence (640–685 nm) by fatty acid substrates and 13S-HPODE. Electron paramagnetic resonance (EPR) of native manganese lipoxygenase showed weak 6-fold hyperfine splitting superimposed on a broad resonance indicating two populations of MnII bound to protein. The addition of linoleic acid decreased both components, and denaturation of the lipoxygenase liberated ∼0.8 Mn²⁺ atoms/lipoxygenase molecule. These observations are consistent with a mononuclear MnII center in the native state, which is converted during catalysis to an EPR silent MnIII state. We propose that manganese lipoxygenase has kinetic and redox properties similar to iron lipoxygenases.

Lipoxygenases oxygenate polyunsaturated fatty acids with one or several (1Z,4Z)-pentadiene units to a hydroperoxy-conjugated diene (1–4). Lipoxygenases are widely distributed in animals and plants, and the lipoxygenase products have a wide range of biological functions as diverse signal molecules, oxidants, and modifiers of membrane structures (1 and 2). Mammalian lipoxygenases insert molecular oxygen at positions C-5, C-8, C-12, or C-15 of arachidonic acid, whereas many plant lipoxygenases insert molecular oxygen at positions C-9 or C-13. All lipoxygenases belong to the same gene family. A characteristic feature is a metal center with non-heme iron (1–4). One lipoxygenase with manganese as a prosthetic metal has been discovered (5, 6). Whether manganese lipoxygenase belongs to the lipoxygenase gene family is presently unknown. Manganese lipoxygenase has so far only been identified in a fungal pathogen of wheat, Geaeumannomyces graminis.

Lipoxygenases with prosthetic iron or manganese have fundamental properties in common but differ in important details. Both types of enzymes abstract with stereospecificity a bisallylic hydrogen from C-3 of the (1Z,4Z)-pentadiene unit and are postulated to form a delocalized alkyl radical over C-1 to C-5 (6–8). Dioxygen reacts with the radical in different ways. All iron lipoxygenases allow oxygen to react with the alkyl radical in an antarafacial way to form a 1-hydroperoxy-(2E,4Z)-pentadiene (1–4, 7), whereas manganese lipoxygenase allows oxygen to react in a suprafacial way at either C-1 or C-3 in a ∼3:1 ratio (6). Manganese lipoxygenase also catalyzes the isomerization of the 3-hydroperoxy-(1Z,3Z)-pentadiene to the end product, the 1-hydroperoxy-(2E,4Z)-pentadiene (6). The differences between iron and manganese lipoxygenases are likely due to steric factors at the active site, which control the position of the alkyl radical and the access of oxygen. The prosthetic iron plays a key role during catalysis. Lipoxygenation starts with oxidation of FeII to FeIII during a short time lag, which is followed by a burst of enzymatic activity and then by steady state catalysis (1–3). The active FeIII-lipoxygenase (FeIII-OH) abstracts the bisallylic hydrogen, an alkyl radical is formed, and FeIII is reduced to FeII (FeII-OH₂). Dioxygen reacts with the radical and forms a peroxyl radical, which regains a hydrogen, the ferrous iron is re-oxidized to ferric (FeIII-OH), and the fatty acid hydroperoxide is formed. Experimental evidence of this mechanism was first provided by EPR analysis of the iron center during soybean lipoxygenase-1 catalysis and by detection of the peroxyl radical (10–12). It is also known that the hydroperoxy group can undergo nonenzymatic rearrangement and be exchanged with surrounding molecular oxygen (13). As an alternative, a lipoxygenation mechanism with an organoiron intermediate has been proposed (14), but many of the described experimental observations are consistent with the radical mechanism (1–3, 10–12).

No information on the prosthetic manganese of manganese lipoxygenase is available except a crude estimate of its manganese content and indirectly by inhibition of the enzyme by a 5-lipoxygenase inhibitor with chelating and reducing properties and by GSH peroxidase (5, 6). Experiments with oxygen-labeled 11S-HPODE and 13R-HPODE and with stereospecifically deuterated linoleic acids have provided a framework

*This work was supported by the Swedish Medical Research Council (6523), the Swedish Society for Medical Research, and the Wallenberg Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: EPR, electron paramagnetic resonance; 11S-HPODE, (11S)-hydroperoxy-(9Z,11E)-octadecadienoic acid; 13R-HPODE, (13R)-hydroperoxy-(9Z,12Z)-octadecadienoic acid; HPLC, high performance liquid chromatography; CHAPS, 3-[N-cyclohexylpropyl]-dimethylammonio-1-propanesulfonic acid; 13R-HPOTrE, 13R-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid; LC-MS, liquid chromatography-mass spectrometry.
for the catalytic mechanism of manganese lipoxygenase described above. However, investigation of the catalytic role of the prosthetic manganese might contribute to elucidation of the reaction mechanism of this enzyme as well as iron lipoxygenases and other dioxygenases. Our main objective was therefore to use a variety of spectroscopic methods to study the kinetics and the metal center of manganese lipoxygenase and to compare the data with other lipoxygenases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fatty acids were obtained as described (5). A mixture of perdeuterated unsaturated fatty acids was purchased from Larodon Fine Chemicals (Malmö, Sweden). Equipment for HPLC, SDS-polyacrylamide gel electrophoresis, and media and columns for chromatography were as described previously in Ref. 5. GSH, soybean lipoxygenase-1 (lipoxigenase type IV), methyl-ω-n-glucopyranoside, and lyophilized glutathione peroxidase (purified as described (15)) were from Sigma. 11S-HPODE and 13R-HPODE were prepared by biosynthesis and characterized by LC-MS (9). 11S-HPODE was enzymatically converted to 13R-HPODE with purified manganese lipoxygenase for quantification by UV at 235 nm.

**Preparation of Manganese Lipoxygenase and Metal Analysis**—Inducingly coupled plasma atomic emission spectroscopy was performed as described (5). Manganese lipoxygenase was purified as described (5) with the following modifications. Fraction I, which eluted with buffer A (10 mM potassium phosphate buffer (pH 6.8), 2 mM NaN$_3$, 0.04% Tween 20) from the phenyl-Sepharose column, was applied to a chelating Sepharose FF column (1.6 × 5.5 cm). The latter was charged with Cu$_2^+$ and equilibrated with 0.2 mM NaCl in buffer A. The run-through fraction was dialyzed against water (dialysis membrane, Union Carbide, Chicago, IL). This material was then pooled with fraction II from the phenyl-Sepharose column and loaded on a CM-Sepharose column. The enzyme eluted with 0.2 M NaCl in buffer A. Active fractions were concentrated (Millipore Ultrafree-15/Biomax-30 centrifugal filter, Bedford, MA) and purified on a Superdex 200 HR column in buffer B (0.05 mM potassium phosphate buffer (pH 7.4), 0.05 mM NaCl, 0.1 mM EDTA, 0.3 mM methyl-$ω$-glucopyranoside, 3 mM NaN$_3$, 1 mM EDTA, 0.5 mM CHAPS). The active fraction had a specific activity ≥8. 2 μM mg$^{-1}$ min$^{-1}$ with linoleic acid as a substrate (25°C) and showed a single band on SDS-polyacrylamide gel electrophoresis. The material was concentrated to 50 mg of protein/ml (Biomax-30 filtration). All glassware was washed overnight with 15% HNO$_3$ and rinsed with Milli-Q water. As an extra precaution, the buffer of the manganese lipoxygenase sample was replaced five times by ultrafiltration with 1 mM EDTA in buffer C (10 mM triethanolamine-HCl (pH 7.3), 2 mM NaN$_3$, 0.04% Tween 20). The run-through of the final Superdex 200 HR column and the flow-through from filtrations were used as controls for EP and atomic emission spectroscopy.

**Manganese Lipoxygenase Assay**—Lipoxygenase activity was assayed by UV and fluorescence monitoring at 25°C (5) and corrected for biosynthesis of 11-hydroperoxy fatty acids without UV absorption at 235 nm as described below. The linear parts of the curves were used to calculate reaction rates using a molar extinction coefficient of 23 m$^2$ cm$^{-1}$ for conjugated diene formation. The biosynthesis of 11S-HPODE and 13R-HPODE was analyzed after mixing manganese lipoxygenase (20 nM) with excess linoleic or [U-2H]linoleic acids (0.25–0.5 mM) in 0.5 ml of buffer (pH 5–11; 0.04% Tween 20 was added to buffers of pH 5, 6, and 7). The steady increase in absorbance (235 nm) was followed until 5–10% of the substrate was metabolized. Vigorous mixing with acidified ethyl acetate stopped the reactions, and the extracted products were analyzed by LC-MS. Enzyme activity was normalized for the protein content determined by total amino acid analysis.

**Amino Acid Analyses**—Manganese lipoxygenase was subject to total amino acid analysis after acidic hydrolysis (6 h HCl, 1 mg/ml phenol, 110°C under vacuum for 24 and 72 h), whereas tryptophane was determined by hydrolysis in alkali (courtesy Dr. D. Eaker of Uppsala Biomedical Center). C-terminal amino acid analysis was performed at the Karolinska Institute, Stockholm (courtesy of Drs. H. Jornvall and E. Cederlund).

**Electronic and Fluorescence Spectroscopy**—Light absorption was recorded as described (5). Fluorescence was recorded with a spectrofluorophotometer (Hitachi F-4000, Tokyo, Japan) with a temperature-controlled cell holder and a magnetic stirrer. Anaerobic incubations were performed with an anaerobic cell repeatedly flushed with argon. Rapid kinetic stopped-flow assays were carried out at 25°C as described (15), and formation of conjugated dienes was recorded at 250 nm (0.2-cm light path; extinction coefficient ~7500 m$^{-1}$ cm$^{-1}$ in buffer C).

**RESULTS**

**Analysis of Amino Acid Residues and Prosthetic Metal**—The amino acid compositions of manganese lipoxygenase, soybean lipoxygenase-1, and human 5-lipoxygenase-1 are shown in Table I. The C-terminal residue of manganese lipoxygenase was valine, whereas the C-terminal residue of most other lipoxygenases is isoleucine (1–3). The apparent molar extinction coefficient at 280 nm was 9.27 × 10$^4$ M$^{-1}$ cm$^{-1}$. The manganese content was determined by atomic emission spectroscopy to be 0.94 mol of manganese/mol of enzyme protein. The iron content was below the detection limit. Concentrated solutions of manganese lipoxygenase were yellow.

**Glutathione Peroxidase**—Soybean lipoxygenase-1 (1 μM/ml with 0.24 mM linoleic acid in buffer C; 25°C) was completely and constantly inhibited by GSH peroxidase (0.1 unit/ml) and 1 mM GSH. In contrast, the reaction rate of manganese lipoxygenase (1.3 μg/ml) was only reduced by 50% with a 10 times larger concentration of GSH peroxidase, and the enzyme re-
The quenching of 13R-HPODE was linear up to 45 °C, which also has been reported for the kinetic isotope effect of manganese lipoxygenase was determined from the mixture. Oleic acid did not quench the long wavelength fluorescence, but linoleic and linolenic acids did as shown by the inset in Fig. 1C. Stopped-flow with 10-s traces was used to calculate the first-order decay rate constants of the fluorescence. The rate constants were 2.3 s⁻¹ and 4.8 s⁻¹ for linoleic and α-linolenic acids, respectively, which were proportional to their turnover numbers.

No quenching was noticed under anaerobic conditions. Preincubation of manganese lipoxygenase with linoleic acid for 30 min under anaerobic condition led to enzyme inactivation, and there was no quenching when aerobic conditions were restored. The long wavelength fluorescence of heat-inactivated enzyme was not quenched by linoleic acid. We conclude that the quench of the long wavelength fluorescence can be related to catalysis of manganese lipoxygenase. Similar findings have also been reported for soybean lipoxygenase-1 (17).

**Formation of HPODE—** Under steady state, linoleic acid was metabolized to 29% 11S-HPODE and 71% 13R-HPODE at pH 7.3. The relative amounts of 11S-HPODE and 13R-HPODE at different temperatures and different pH values were determined. Temperature changes from 15 to 45 °C were without apparent effect but pH changes had a marked influence. Biosynthesis of 11S-HPODE increased from 3% at pH 5, to 29% at pH 7 and reached 40% at pH 11 (Fig. 2). As previously reported, manganese lipoxygenase has a broad pH optimum centered at pH 7 with over 60% enzyme activity at pH 5 and at pH 11 (5). 11S-HPODE is chemically unstable at acidic pH (6), but this could not explain the low formation at pH 5, as 11S-HPODE was found to be chemically stable under these conditions. However, linoleic acid has a pKₐ between pH 7 and 8 (1), and the charge of the carboxyl group may affect substrate binding and the biosynthesis of products.

Oxidation of [11S-²H]linoleic acid by manganese lipoxygenase occurred with a large kinetic isotope effect, k_D/k_S = 15–22 (6). UV and LC-MS analysis showed that [U⁻²H]linoleic acid was oxidized by manganese lipoxygenase and by soybean lipoxygenase-1 with a similar kinetic isotope effect. The kinetic isotope effect of manganese lipoxygenase was determined from duplicate determinations at 25 °C to yield k_D/k_S = 21–24 (with correction for 11S-HPODE formation), whereas soybean lipoxygenase-1 yielded k_D/k_S = 20–23. The kinetic isotope effect of manganese lipoxygenase was not temperature-dependent (5–45 °C), which also has been reported for the kinetic isotope effect of soybean lipoxygenase-1 (17).

**Fluorescence of Manganese Lipoxygenase—** The emission spectrum of manganese lipoxygenase during excitation at 280 nm is shown in Fig. 1A. Soybean lipoxygenase-1 gives a similar emission spectrum. The fluorescence of manganese lipoxygenase centered at 345 nm decreased in the presence of fatty acids, which were not substrates of manganese lipoxygenase. The long wavelength fluorescence of heat-inactivated enzyme occurred with a large kinetic isotope effect, k_D/k_S = 15–22 (6). UV and LC-MS analysis showed that [U⁻²H]linoleic acid was oxidized by manganese lipoxygenase and by soybean lipoxygenase-1 with a similar kinetic isotope effect. The kinetic isotope effect of manganese lipoxygenase was determined from duplicate determinations at 25 °C to yield k_D/k_S = 21–24 (with correction for 11S-HPODE formation), whereas soybean lipoxygenase-1 yielded k_D/k_S = 20–23. The kinetic isotope effect of manganese lipoxygenase was not temperature-dependent (5–45 °C), which also has been reported for the kinetic isotope effect of soybean lipoxygenase-1 (17).
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Fig. 3. Stopped-flow analysis of manganese lipoxygenase with α-linolenic acid and with 11S-HPODE. A, a 20-s kinetic trace, which shows the increase in absorption at 250 nm due to formation of 13R-HPOTrE from 35 μM α-linolenic acid. The inset shows a 0.5-s kinetic trace (mean of three shots). B, a 20-s kinetic trace, which shows the increase in absorption at 250 nm due to formation 13R-HPODE from 13 μM 11S-HPODE. The inset shows a 0.5-s kinetic trace (mean of three shots). 1 μM manganese lipoxygenase was used in all experiments.

effect of soybean lipoxygenase-1 (18). [U-2H]Linoleic acid was metabolized by manganese lipoxygenase to 15 and 85% of [U-2H]13S-HPODE and [U-2H]13R-HPODE, respectively, during steady state (pH 7.3), possibly because of steric effects of the perdeuterated substrate.

Kinetic Analysis—Stopped-flow with kinetic traces of 0.5 and 20 s was used to study the oxygenation of α-linolenic acid to 13R-HPOTrE by manganese lipoxygenase. The 20-s trace showed that the reaction rates increased for a few seconds, reached steady state, and then leveled off due to the exhaustion of substrate (Fig. 3A). The 0.5-s trace demonstrated that catalysis started after a lag phase of ~40 ms, which was followed by a burst of enzyme activity for ~55 ms and then by steady state catalysis (Fig. 3A, inset). The $k_{cat}$ for oxidation of α-linolenic acid to 13R-HPOTrE at 25 °C was calculated to be 47 s$^{-1}$. As previously reported, α-linolenic acid had the highest turnover of the unsaturated C18 fatty acids and the lowest $K_m$ (2.2 μM) (5).

Stopped-flow with linoleic acid yielded similar traces, but linoleic acid was metabolized at only half the rate of α-linolenic acid. Quantitative stopped-flow data with linoleic acid and with 11S-HPODE are summarized in Table II. The lag phase of linoleic acid was not reduced by preincubation of manganese lipoxygenase with 13R-HPODE (Table II). 11S-HPODE was also converted to 13R-HPODE after a lag phase, which was followed by a relatively long lasting burst of enzyme activity and then by steady state biosynthesis at a perfectly linear rate (Fig. 3B). 11S-HPODE was metabolized to 13R-HPODE at less than half the rate of linoleic acid oxidation (Table II).

Using conventional spectroscopy, the $K_m$ and $V_{max}$ for isomerization of 11S-HPODE were found to be 8.1 μM and 7.5 μmol min$^{-1}$ mg$^{-1}$, respectively, at pH 7.3 and 25 °C. The $V_{max}$ corresponded to $k_{cat} = 9.1$ s$^{-1}$.

EPR Analysis—EPR spectra of manganese lipoxygenase (0.6 mM, 100 K) revealed 6-fold hyperfine splitting on a broad singlet (Fig. 4, trace a). Protein-bound Mn$^{II}$ characteristically shows weak EPR signals (19). The signals were not due to unbound Mn$^{II}$ for two reasons. First, atomic emission spectroscopy of the buffer, which was obtained by filtration of the enzyme, contained no detectable manganese. Second, EPR analysis was performed after mixing this enzyme preparation with 4 mM linoleic acid. The reaction was quenched (~105 °C) after incubation for ~1 s and for 10 min at room temperature. The EPR spectra after 1 s now showed that the 6-fold hyperfine splitting had almost disappeared together with a prominent decrease of the broad singlet and that a weak radical signal appeared at g = 2.005 (Fig. 4, trace c). After 10 min the Mn$^{II}$ characteristics had completely disappeared, and the radical at g = 2.005 had decreased to 1/3 of the initial value (Fig. 4, trace c). EPR analysis with 11S-HPODE as a substrate yielded essentially similar results, i.e. the Mn$^{II}$-related signals decreased and the radical signal was apparent. The manganese signal of the native enzyme was therefore likely due to two different populations of Mn$^{II}$, one with octahedral coordination and the other with tetrahedral coordination (20), which were oxidized during catalysis to an EPR silent Mn$^{III}$ state. The radical signal showed peak to trough width of 1.0 millitesla centered at g = 2.005. The saturation behavior indicated that it was not interacting with any magnetic relaxing species. The quantity of the radical before and 1 s after the addition of substrates yielded similar results, about 0.3 μM or 0.05% of the enzyme concentration, but decreased to about 0.1 μM 10 min after the addition of linoleic acid. The radical was not present in the buffer (Fig. 4, trace d). It seems possible that the disappearance of the six manganese signals made this signal appear (cf. traces a and b of Fig. 4). The nature of the radical was not further investigated.

The transition from an Mn$^{II}$ to an Mn$^{III}$ center during catalysis was obvious from the color of the EPR samples, which were yellow in the resting state and colorless in the presence of
bound metal and increased the manganese signals. The EPR spectrum of manganese lipoxygenase (0.1 mM) after denaturation of the protein with 15% HNO₃, which released the amount of Mn²⁺ ions is bound in the tetrahedral coordination.

Manganese lipoxygenase was also analyzed by EPR after denaturation of protein with 15% HNO₃, which released the bound metal and increased the manganese signals. The EPR spectrum exhibited a nearly isotropic g = 2.0 signal having 6-fold hyperfine splitting (A = 9.5 millitesla) typical of octahedrally coordinated Mn²⁺ in solution (Fig. 4, trace e) (19). This spectrum was identical with an aqueous standard of MnCl₂. The amount of Mn²⁺ in the denatured sample was estimated from spin quantitation to be 0.8 ± 0.08 atoms/lipoxygenase molecule. This was slightly lower than the manganese content according to atomic emission spectroscopy.

**DISCUSSION**

The oxygenation of linoleic acid by manganese lipoxygenase and iron lipoxygenases differs with respect to the stereochemistry of hydrogen abstraction and oxygen insertion at C-11 and/or C-13 of the delocalized alkyl radical, but the metal centers of both manganese and iron enzymes were nevertheless found to be mechanistically similar. Soybean lipoxygenase-1 and human 5-lipoxygenase contain a mononuclear Fe³⁺ center, which is oxidized to Fe⁴⁺ in the active enzyme (1–3, 10–11, 21). Atomic emission spectroscopy and EPR now demonstrate that manganese lipoxygenase also contains a mononuclear metal center. EPR of resting manganese lipoxygenase was consistent with two different populations of Mn³⁺ bound to the apoprotein in tetrahedral and octahedral coordination (19, 20). The EPR signals of Mn⁴⁺ decreased during catalysis suggesting that the metal was oxidized to a nonparamagnetic state, presumably Mn⁵⁺. The Mn³⁺ oxidation state is a common form of manganese in biological systems and Mn⁵⁺ ⇔ Mn⁶⁺ redox cycling is observed in superoxide dismutase and catalase (22).

To obtain readily detectable signals with EPR we used 600-fold higher concentration of manganese lipoxygenase than during stopped-flow, which might influence the comparison of EPR spectra with stopped-flow kinetics. Nevertheless, the oxidation of Fe⁴⁺ and Mn³⁺ in lipoxygenases might be related to the lag phase, the inhibitory effect of GSH peroxidase (although it had a relatively weak and transient inhibitory effect on manganese lipoxygenase), and to inhibition of manganese lipoxygenase with a lipoxygenase inhibitor with reducing properties, BW44C (5). A hypothetical reaction mechanism of manganese lipoxygenase is outlined in Fig. 5.

The turnover of α-linolenic and linoleic acids by manganese lipoxygenase is ~47 and ~26 s⁻¹, respectively, whereas the turnover of linoleic acid by soybean lipoxygenase-1 is ~260 s⁻¹ (23). A peroxy radical can be readily detected by EPR during soybean lipoxygenase catalysis, and an alkyl radical has been tentatively identified (12). According to isotope experiments (6), these radicals are also likely formed both during oxidation of linoleic acid and isomerization of 11S-HPODE to 13R-HPODE by manganese lipoxygenase. Substrate-induced peroxy or alkyl radicals could not be detected by EPR under our experimental conditions. It is conceivable that these transient radicals were formed in too low a concentration due to the relatively low turnover of manganese lipoxygenase. The origin of the weak radical signal we observed at g = 2.005 is unclear.

The initial step of manganese lipoxygenase and iron lipoxygenase catalysis is removal of the pro-S hydrogen at C-11, which is accompanied by a large isotope effect (kDs/kD1 > 15 for manganese lipoxygenase (6) and kDs/kD1 = 20–80 for soybean lipoxygenase-1 (8, 18, 24)). The theoretically expected value of kDs/kD1 is 7–10 (18). Perdeuterio linoleic acid yielded a similar kinetic isotope effect as [11S-²H]linoleic acid, which implies a small secondary isotope effect. The reason for the large primary kinetic isotope effect is unknown but might be related to proton leakage through a potential barrier (18). The de Broglie wavelengths are 0.5 Å for ¹H and 0.3 Å for ²H at 10 kJ mol⁻¹, and the probability of proton tunneling is a function of these wave-lengths and the barrier width (25, 26). When hydrogen abstraction has occurred, 11S-HPODE and 13R-HPODE are formed in parallel in a pH-dependent ratio. It is interesting to compare the rate of biosynthesis of 11S-HPODE and 13R-HPODE from linoleic acid and the rate for isomerization of 11S-HPODE to 13R-HPODE (Table II). The data suggest that biosynthesis of 11S-HPODE from linoleic acid and the isomerization of 11S-HPODE to 13R-HPODE occur at a lower rate (kcat = 7 and 9

**Fig. 4.** EPR spectra of manganese lipoxygenase. Trace a shows the EPR spectrum of native manganese lipoxygenase. Trace b shows an EPR spectrum of manganese lipoxygenase incubated with linoleic acid for 1 s. Trace c shows EPR spectrum of manganese lipoxygenase incubated for 10 min with linoleic acid. Manganese lipoxygenase (0.6 mM) and linoleic acid (4 mM; or solvent only) were incubated for 1 s and 10 min and then immediately quenched by rapid freezing (−105 °C). Trace d shows EPR spectrum of a buffer B blank from the last purification step of manganese lipoxygenase. Trace e shows EPR spectrum of manganese lipoxygenase (0.1 mM) after denaturation of the protein with 15% HNO₃, which released the amount of Mn²⁺ ions is bound in the tetrahedral coordination.

**Fig. 5.** A hypothetical reaction mechanism of manganese lipoxygenase. Resting enzyme is in the Mn⁰ oxidation state and oxidized to the active form, Mn⁴⁺, which is proposed to undergo redox changes as indicated. The alkyl and peroxy radicals within brackets have not been identified but are likely formed according to isotope experiments (6). Glutathione peroxidase (GSH-Px) inhibits the reaction. The 3-hydroperoxypentadiene can be isomerized to the cis-trans conjugated hydroperoxypentadiene end product by the enzyme (as indicated by the arrows).
whereas FeII can be easily oxidized in the aerobic world. The
lipoxygenase is unknown. In oxidation state III of manganese
We can only speculate, as the biological function of manganese
lipoxygenase belongs to the lipoxygenase gene family.

It will be of phylogenetic and
ids, showed that only substitution with valine retained signif-
icant lipoxygenase activity (29). It will be of phylogenetic and
mechanistic interest to determine whether manganese lipox-
genase belongs to the lipoxygenase gene family.

Are there any advantages with a manganese lipooxygenase?
We can only speculate, as the biological function of manganese lipooxygenase is unknown. In oxidation state III of manganese and iron lipooxygenases, the two metals are expected to be similar in binding and other physicochemical properties (30). In oxidation state II of resting enzymes, however, the two metals may differ significantly. MnIII is chemically stable, whereas FeIII can be easily oxidized in the aerobic world. The different activation properties observed for manganese lipooxygenase and soybean lipooxygenase-1 may reflect the different redox potentials for transition from oxidation state II to oxidation state III. Manganese lipooxygenase is secreted by

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s−1, respectively) than the biosynthesis of 13R-HPDE (kcat = 19 s−1) from linoleic acid.
