Structural Characterization of Alkyl and Peroxyl Radicals in Solutions of Purple Lipoxygenase‡

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ABSTRACT: Soybean lipoxygenase isozyme 1 catalyzes the addition of dioxygen to fatty acid substrates that contain a 1,4-diene, generating allylic hydroperoxides. EPR spectra of purple enzyme solutions, formed by addition of saturating amounts of substrates or product to the enzyme, reveal the existence of fatty acid alkyl and peroxyl radicals that are bound to the enzyme and may be intermediates of the catalytic reaction [Nelson, M. J., Seitz, S. P., & Cowling, R. A. (1990) *Biochemistry 29*, 6897–6903]. We have analyzed the spectra of the radicals formed from the hydroperoxide products of four specifically deuterated linoleic acids and [per-^{2}H]linoleic acid. The alkyl radical is an allyl radical, delocalized over C9 through C11 of linoleic acid. The data are consistent with delocalization of some of the spin over an unknown substituent at C12. The peroxyl radical is a 9-peroxyl derivative of linoleic acid. From the data we propose a novel mechanism for the lipoxygenase reaction: (1) oxidation of the 1,4-diene by the active-site Fe^{3+} to a Δ^{12}-[9,10,11]-allyl radical; (2) activation of dioxygen at the Fe^{2+}; (3) electrophilic attack by Fe^{2+}–O_2 on the 12-ene to form a 12,13-perepoxy-[9,10,11]-allyl radical; (4) opening of the perepoxide to the Fe^{3+}–allylic hydroperoxide complex; (5) protonation to yield the 13-hydroperoxide. Addition of dioxygen to the allyl radical is proposed to form the 9-peroxyl, ultimately to yield the minor 9-hydroperoxide lipoxygenase product.

Lipoxygenases are non-heme iron enzymes that catalyze the addition of dioxygen to polyunsaturated fatty acids. Soybean lipoxygenase isozyme 1 catalyzes the transformation of linoleic acid to a mixture of 13(*S*)-hydroperoxy-9,11-(*E*,*Z*)-octadecadienoic acid [13(*S*)-HPOD[1]] and 9(*R*,*S*)-hydroperoxy-10,12-(*Z*,*E*)-octadecadienoic acid (9-HPOD) (Hamburg & Samuelsson, 1967; Gardner, 1989; Nikolaev et al., 1990). Under certain conditions the enzyme is highly selective for the production of 13(*S*)-HPOD. The plant growth regulators jasmonic, traumatic, and abscissic acids are synthesized from lipoxygenase products (Gardner, 1991; Siedow, 1991). In mammals the fatty acid hydroperoxides are substrates for the synthesis of leukotrienes and lipoxins, species with broad inflammatory activity (Samuelsson et al., 1987; Wasserman et al., 1991). Consequently, inhibitors of lipoxygenases with antiinflammatory activity have been a major goal of the pharmaceutical industry (Batt, 1992; McMillan & Walker, 1992).

Any chemical mechanism for the reaction catalyzed by lipoxygenase must take into account certain key observations. (1) In the absence of dioxygen, linoleic acid reduces the active-site iron from Fe^{3+} to Fe^{2+} in a reaction that occurs rapidly enough to be a step in the enzymatic reaction (De Groot et al., 1975b; Egmond et al., 1977). These reactions generate fatty acid radicals that diffuse out of the active site and have been identified as adducts with spin traps (Garssen et al., 1972; de Groot et al., 1973). (2) There is a large primary deuterium kinetic isotope effect observed using [11(*pro-S*)-^{2}H]linoleic acid, suggesting that the 11(*pro-S*) C–H bond is broken in the rate-determining step (Egmond et al., 1973). A secondary isotope effect has been seen upon substitution of all of the vinylic protons of the substrate (Wiseman, 1989), suggesting that this C–H bond cleavage results in a radical or anion that is delocalized over one or both of the adjacent double bonds. (3) The 9*E*,12*Z* analogue of linoleic acid is a substrate for lipoxygenase, but yields the 9*Z*,11*E* fatty acid hydroperoxide product (Funk et al., 1987). This requires that the Δ^9 double bond be sufficiently weakened to allow rotation at some step of the mechanism, again implying delocalization of an intermediate anion or radical over at least the Δ^9 double bond.

These data are consistent with a mechanism involving activation of the fatty acid via oxidation by the non-heme ferric ion in the active site to generate a fatty acid alkyl radical. This may react directly with dioxygen, producing a fatty acid peroxyl radical which is reduced by the (now) ferrous ion (De Groot et al., 1975b). The structure proposed for the intermediate fatty acid alkyl radical is the most stable one-electron oxidation product of linoleic acid, a pentadienyl radical. An inherent difficulty with this structure is that dioxygen would be expected to react at both the 9 and 13 positions, on the *pro-R* and *pro-S* faces, ultimately generating four regio- and stereoisomeric hydroperoxides (Porter, 1986). The regio- and stereoselectivity exhibited by lipoxygenases would have to be enforced either by steric hindrance to the approach of dioxygen to all but one face and one end of the radical, by discrimination in the rates of reduction of the various peroxyl radicals generated, or by direction of the reaction by coordination of the dioxygen to the iron before it reacts with the intermediate. In support of a radical mechanism, fatty acid alkyl and peroxyl radicals have been detected directly by EPR in solutions of lipoxygenase and substrate under turnover conditions (Chamulitrat & Mason, 1989). However, the mechanistic com-

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[1] Abbreviations: 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9,11-(*E*,*Z*)-octadecadienoic acid; 9-HPOD, 9(*R*,*S*)-hydroperoxy-10,12-(*Z*,*E*)octadecadienoic acid.

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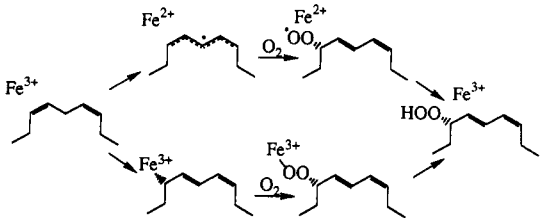
petence of these species has not been established.



A second mechanism that is consistent with the experimental observations is one in which the fatty acid is activated by coordination of the ferric ion to C13 with concomitant deprotonation at C11 (Corey & Nagata, 1987). Insertion of dioxygen into the iron–carbon bond and heterolytic cleavage of the resulting ferric peroxide complex would yield the product. This mechanism has the advantage of predicting directly the regio- and stereospecificity of the reaction. It rationalizes the observed radical production as the result of slow Fe–C bond homolysis of the organometallic intermediate, which would compete with insertion at low dioxygen concentrations. It does not, however, explain the production of 9(*E*) product from 9(*Z*) substrate, except as occurring via a bound fatty acid radical that re-forms the Fe–C bond after isomerization.

One way to distinguish between the proposed mechanisms is to trap and characterize intermediates of the reaction. So far, only one spectroscopically distinct species formed from the reaction between lipoxygenase and linoleic acid has been shown to be kinetically competent to be an intermediate, and that species has not been characterized structurally (de Groot et al., 1975a; Egmond et al., 1977; Spaapen et al., 1979). It has a visible absorbance maximum at approximately 585 nm, giving the enzyme solution a purple color. These solutions also exhibit an EPR signal at $g = 4.3$ (Slappendel et al., 1983), associated with high-spin Fe^{3+} in a rhombic electronic environment, but the connection between the purple color and the EPR spectrum is circumstantial. Recently two synthetic Fe^{3+}–alkyl peroxide complexes were reported to have visible spectra similar to that of purple lipoxygenase (Nishida & Akamatsu, 1991; Zang et al., 1993), suggesting that the purple enzyme species might be a Fe^{3+}–fatty acid peroxide complex, possibly the penultimate intermediate of the enzyme reaction.

We have examined frozen solutions of purple lipoxygenase by EPR spectroscopy, operating under the hypothesis that this purple complex, whatever its structure, is an intermediate of the lipoxygenase reaction and that the purple solutions may contain other intermediates. We have reported finding both a peroxyl radical and an alkyl radical in these samples, depending on the concentration of dioxygen present (Nelson & Cowling, 1990; Nelson et al., 1990). Unfortunately, the turnover number of lipoxygenase (approximately 300 s^{-1}) is too rapid to allow determination of the kinetic competence of these species by stopped-flow (freeze–quench) EPR spectroscopy. We report structural information obtained by studying the effects of specific deuterium substitution on the EPR spectra of the alkyl and peroxyl radicals, and discuss the potential mechanistic relevance of these species.

MATERIALS AND METHODS

The [per-^{2}H]linoleic acid (98% enriched) was obtained from Cambridge Isotopes, deuterium gas (99% enriched) from MSD Isotopes, and $^{17}O_2$ gas (23% enriched) from Mound Laboratories. Linoleic acid was purchased from Aldrich Chemicals, further purified by chromatography on silica gel (hexane/ethyl acetate/acetic acid, 65:34:1), and stored frozen at −80 °C. Methyl crepenynoate was the generous gift of Dr. Glenn L. Ford, Food Research Laboratory, CSIRO. Other chemicals were obtained from Aldrich Chemicals.

Synthesis of Specifically Deuterated Linoleic Acids. [11,11-^{2}H]-, [8,8-^{2}H]-, and [9,10-^{2}H]linoleic acids were prepared by a route involving alkylation of the bis-magnesium salt of 9-decynoic acid with 1-bromooct-2-yne or 1-bromooct-2(*Z*)-ene followed by reduction of the resulting alkynes (Osbond et al., 1961). For the synthesis of [8,8-^{2}H]linoleic acid, deuterium was introduced by sodium borodeuteride reduction of the mixed anhydride formed from monomethyl suberate and ethyl chloroformate. Reaction of the resulting methyl [8,8-^{2}H]-8-hydroxyoctanoate with hydrobromic acid gave the bromo acid, which was treated with lithium acetylide–ethylenediamine complex in dimethyl sulfoxide to give the required [8,8-^{2}H]-9-decynoic acid. This was coupled with 1-bromooct-2-yne and the product diyne esterified with diazomethane and reduced with H_2 over quinoline-poisoned Lindlar catalyst to yield methyl [8,8-^{2}H]linoleate. The 9,10-^{2}H-labeled analog was prepared by coupling natural abundance 9-decynoic acid with 1-bromooct-2(*Z*)-ene followed by methyl ester formation with diazomethane and reduction with $^2H_2$ over poisoned Lindlar catalyst. The 11,11-^{2}H-labeled analog was prepared similarly, according to the literature (Tucker et al., 1971). Methyl [12,13-^{2}H]linoleate was prepared from methyl octadec-9(*Z*)-en-12-ynoate (methyl crepenynoate) by reduction with $^2H_2$ gas over poisoned Lindlar catalyst. The undesirable isomers of the alkenes were removed by chromatography on silver nitrate impregnated silica gel [30% (v/v) ether/hexane], and the fatty acids were prepared by saponification with lithium hydroxide. All compounds were homogeneous by thin-layer chromatography and exhibited analytical data (^{1}H-NMR, ^{13}C-NMR, IR, CI-MS) consistent with their proposed structures. 13(*S*)-HPOD was synthesized from natural abundance and deuterated linoleic acids, using natural abundance and 23% enriched $^{17}O_2$, as previously described (Nelson et al., 1990).

Sample Preparation. Soybean lipoxygenase 1 was purified and assayed as previously described (Nelson, 1988). The specific activities of the samples used ranged from 200 to 230 units/mg. The active-site iron was oxidized by titration with 13(*S*)-HPOD in 0.05 M borate, pH 9.0, until the first appearance of absorption at 585 nm indicated that the oxidation was complete. The oxidized protein was dialyzed vs two changes of 0.05 M borate, pH 9.0, to remove excess 13(*S*)-HPOD as well as the organic products of the oxidation reaction. The enzyme solutions were equilibrated against either argon or dioxygen in a serum vial, then transferred to a stoppered cuvette containing the same atmosphere, and placed in the cuvette holder of a Hewlett-Packard HP 8451a diode array spectrophotometer. The cuvette holder was kept at 4 °C by a circulating water bath. Purple samples were prepared by addition of a 10-fold excess of the appropriate linoleic acid or 13(*S*)-HPOD, the visible spectrum was verified, and a gas-tight syringe was used to transfer the sample to a chilled, stoppered EPR tube containing the same atmosphere. The sample was immediately frozen in liquid nitrogen.

EPR Spectroscopy. EPR spectra were obtained using a Brucker EM-200 with an Oxford Instruments ESR900 liquid helium cryostat operating at 30 K. The conditions were as follows: microwave frequency, 9.42 GHz; modulation amplitude, 0.3 mT; sweep width, 20 mT; sweep time, 0.12 mT/s; 1000 data points taken. For spectra of the alkyl radical, the

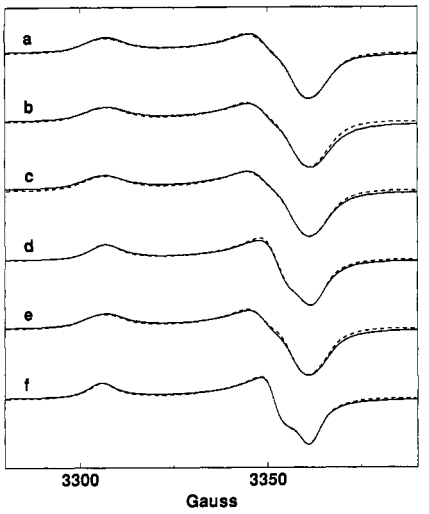


FIGURE 1: EPR spectra of specifically deuterated peroxyl radicals in purple lipoxygenase. Prepared from (a) natural abundance linoleic acid, (b) [12,13-^{2}H]linoleic acid, (c) [11,11-^{2}H]linoleic acid, (d) [9,-10-^{2}H]linoleic acid, (e) [8,8-^{2}H]linoleic acid, and (f) [per-^{2}H]linoleic acid; (—) data; (- - -) simulation using g values and hyperfine couplings in Table 1.

microwave power was set to 1 mW, and for spectra of the peroxyl radical, 4 mW. EPR simulations were carried out using XPOW (Altman, 1981; Nilges, 1981; Maurice, 1982; Duliba, 1983) running on a Macintosh computer.

RESULTS

Treatment of solutions of ferric lipoxygenase (10–25 mg/mL) at pH 9 with an excess of either linoleic acid or 13(S)-HPOD leads to the formation of a purple color that persists for several minutes at 4 °C. Frozen solutions made in this way evince EPR spectra of alkyl and/or peroxyl radicals, depending on the concentration of dioxygen in the sample (Nelson & Cowling, 1990; Nelson et al., 1990). The EPR spectra of peroxyl radicals generated from four specifically deuterated linoleic acids and from [per-^{2}H]linoleic acid are shown in Figure 1. There is a substantial difference in the line shapes of the peroxyl radicals generated from natural abundance and [per-^{2}H]linoleic acids, suggesting unresolved hyperfine splitting by protons on the fatty acid backbone. Only treatment with [9,10-^{2}H]linoleic acid, among the specifically labeled substrates used, leads to an EPR spectrum with a line shape similar to that of the [per-^{2}H]peroxyl. Thus the peroxyl radical we are observing is either a 9- or 10-peroxyl derivative of linoleic acid.

Each of these spectra has been simulated, starting with the spectrum of the peroxyl radical generated from [per-^{2}H]-linoleic acid. Using line widths and g values from that simulation, the spectra of the peroxyl derivatives of [8,8-^{2}H]- and [9,10-^{2}H]linoleic acids were simulated. This allowed an estimate of the coupling to the peroxyl radical from protons at the 8, 9, and 10 positions of the fatty acid (Table 1). In the simulations the hyperfine couplings from protons on C8 and C10 were assumed to be isotropic, but a similar restriction on the hyperfine coupling from the proton at C9 prevented successful simulation of the spectra. (It should be noted that the lack of resolved hyperfine splitting in the g_3 = 2.035 and g_1 = 2.0017 features of the peroxyl radical spectra results in the magnitudes of A_1 and A_3 determined for the protons on C8, C9, and C10 being highly correlated.) The parameters obtained from these simulations were then used to generate a satisfactory simulation of the EPR spectrum of the natural abundance peroxyl radical. Observation of weak hyperfine

Table 1: Hyperfine Coupling Constants for Protons on Alkylperoxyl and Allyl Radicals Arising from Linoleic Acid in Solutions of Purple Lipoxygenase[a]

	peroxyl			allyl		
	A_1	A_2	A_3	A_1	A_2	A_3
H_8	0.26[b]	0.30	0.27	1.39	1.39	1.39
H_8'				0.46	0.46	0.46
H_9	0.49	0.78	0.49	1.81	0.60	1.21
H_{10}	0.28	0.28	0.28	[c]	[c]	[c]
H_{11}	[d]	[d]	[d]	1.54	0.51	1.03
$H_{12,13}$	[d]	[d]	[d]	[d]	[d]	[d]

[a] A_i (mT ± 0.05) corresponds to g_i, where for the alkylperoxyl radical g_1 = 2.0017, g_2 = 2.0075, and g_3 = 2.0353 and for the allyl radical g_1 = 2.0033, g_2 = 2.0038, and g_3 = 2.0030. [b] Spectra could be fitted equally well assuming one proton with these couplings or two protons with smaller couplings. [c] Not determined. [d] Small; substitution by ^{2}H leads to no detectable effect on the EPR spectrum.

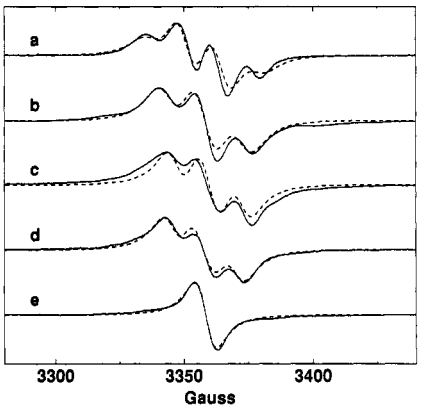


FIGURE 2: EPR spectra of specifically deuterated alkyl radicals in purple lipoxygenase. Prepared from (a) 13-HPOD, (b) [11-^{2}H]-13-HPOD, (c) [9,10-^{2}H]-13-HPOD, (d) [8,8-^{2}H]-13-HPOD, and (e) [per-^{2}H]-13-HPOD; (—) data; (- - -) simulation using g values and hyperfine couplings in Table 1.

coupling to one or both protons on C8, together with the observation of relatively strong coupling to the proton on C9 or C10, demonstrates that the species we are observing is the 9-peroxyl derivative of linoleic acid. The spectra of the peroxyl radicals generated from [11,11-^{2}H]- and [12,13-^{2}H]linoleic acid and natural abundance linoleic acid are superimposable; thus, there is no detectable 13-peroxyl radical in the samples.

Purple lipoxygenase solutions generated from 13(S)-HPOD under anoxic conditions contain alkyl radicals. We see the same alkyl radical EPR spectra in samples prepared using linoleic acid and limiting dioxygen, but spectra of those samples often have additional contributions from the 9-peroxyl radical and are thus more difficult to interpret. Consequently, the analysis that follows is based on spectra of samples prepared with 13(S)-HPOD and deoxygenated solutions of lipoxygenase.

The EPR signal of the alkyl radical generated using natural abundance 13(S)-HPOD is a quartet, suggesting partially resolved hyperfine splitting by three protons. We have shown previously that one of those is the proton on C11 of 13(S)-HPOD by specific labeling with deuterium (Nelson et al., 1990). We have extended that experiment using [8,8-^{2}H]-, [9,10-^{2}H]-, [12,13-^{2}H]-, and [per-^{2}H]-13(S)-HPOD. EPR spectra of these radicals are shown in Figure 2. The spectrum of the radical generated from the [per-^{2}H]-13(S)-HPOD is a singlet, demonstrating that all of the protons that are strongly coupled to the radical are part of the fatty acid backbone. This experiment and its complement, using natural abundance

13(*S*)-HPOD to generate purple lipoxygenase samples in ^2H_2O (data not shown), eliminate the possibility that any of the strongly coupled protons might be in exchange with water at some point during formation of the radical. Substitution of the protons at C12 and C13 with deuterium has no significant effect on the EPR spectrum of the alkyl radical. The spectra of the radicals generated with [8,8-^{2}H]- and [9,10-^{2}H]-13-(*S*)-HPOD are triplets, showing that one of the three strongly coupled protons is on C8, and one on either C9 or C10. Combined with our previous work, this completes the assignment of the three strongly coupled protons to those on C11, C9 or C10, and one of the protons on C8, and allows us to assign the structure to an allyl radical delocalized over C9, C10, and C11.

Simulations of all of the spectra in Figure 2 were used to generate a self-consistent set of hyperfine coupling tensors for the radical. The starting point for the simulation was the set of *g* values and hyperfine coupling tensors of allyl radical studied in an argon matrix (Maier et al., 1983). As above, the intrinsic line widths were set using the spectrum of the radical generated using the [per-^{2}H]-13(*S*)-HPOD, which shows no resolved hyperfine splittings. Then the individual spectra of the radicals generated using the specifically deuterated 13(*S*)-HPODs were simulated. It was not necessary to alter the *g* values during the course of the simulations; however, the hyperfine tensors of the unsubstituted allyl radical (Maier et al., 1983) were much too large in magnitude to fit our data. In adjusting the proton couplings to fit the data we kept the same direction and relative magnitudes of the components as reported (Maier et al., 1983): for the protons on C9 and C11, A_y:A_z:$A_x \approx$ 1:2:3, and for the proton on C10, A_z:A_y:$A_x \approx$ 1:2:3. Couplings from the protons on C8 were assumed to be isotropic. The derived hyperfine coupling constants are shown in Table 1.

The magnitude of the dipolar couplings of the protons on C8 is a function of the orientation of those protons with respect to the plane of the allyl radical. We estimated the dihedral angle θ by the following logic: for a methyl proton of the ethyl radical (i.e., adjacent to a unit spin) the magnitude of the hyperfine coupling is $0.4 + 5.0 \cos^2 \theta$ mT, where θ is the dihedral angle between the relevant C–H bond and the p orbital that contains the electron (Morton, 1964). We adapted this equation by reducing it by the ratio of the spin density on C9 to the spin density on a methyl radical. This we took as the ratio of the effective isotropic hyperfine coupling of the proton on C9 (estimated to be 1.2 mT)[2] and methyl radical (2.3 mT) (McConnell & Strathdee, 1959). Thus, the equation we used for the splittings from the protons on C8 was $A_{iso} = 0.21 + 2.6 \cos^2 \theta$ mT. Using this equation, $\theta = 48°$ for the strongly coupled proton on C8. The dihedral angle for the other proton on C8 then is $\theta \pm 120°$. Only $\theta' = 288°$ predicts an unresolvably small value for the hyperfine splitting from that proton, consistent with the data.

DISCUSSION

We have formulated the hypothesis that the conditions used for the formation of purple lipoxygenase allow the enzyme–product complex to partition among the several enzyme–intermediate species. Under this hypothesis, the purple species is one of these enzyme–intermediate complexes. We have reported EPR spectra of an alkyl and a peroxyl radical in frozen solutions of purple lipoxygenase at cryogenic temperatures (Nelson & Cowling, 1990; Nelson et al., 1990). As a test of our hypothesis we report the partial determination of the structures of these radicals in order to investigate their chemical competence as intermediates.

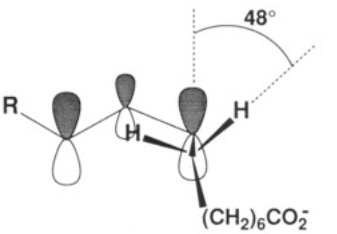


FIGURE 3: Predicted structure of the allyl radical formed in solutions of purple lipoxygenase. The structure formed by rotation about the C8–C9 bond by 180° fits the data equivalently.

Structure and Origin of the Alkyl Radical. The alkyl radical is not the most stable one-electron oxidation product of linoleic acid, a pentadienyl radical, as we initially proposed (Nelson et al., 1990). Instead it is an allyl radical derived from linoleic acid, with the unpaired electron delocalized over carbons 9–11. From the magnitude of the dipolar coupling to the single strongly coupled proton on C8, we have calculated the dihedral angle between the π orbital that contains the unpaired electron and the C–H bond of that proton to be 48°. Thus we know the geometry of the carbon skeleton of this radical from C7 to C12 (Figure 3).

The hyperfine couplings observed for the protons on C9 and C11 are 0.2–0.3 mT smaller than those seen in neutral, alkyl-substituted allyl radicals (Griller et al., 1984). One explanation for the small hyperfine couplings seen in the spectra is that the radical we observe is not a neutral allyl radical. Radical anions typically display smaller proton hyperfine couplings than do analogous neutral radicals (Bolton, 1965); however, there appears to be no reasonable mechanism for the generation of a radical anion in this system. Another explanation is a substituent on either C9 or C11 over which some of the spin has been delocalized (Griller et al., 1984). It seems reasonable to conclude that the substituent would be at the C11 end of the radical because the fatty acids used to generate the radicals are functionalized at that position (e.g., with a 2-alkenyl substituent in linoleic acid).

One structure superficially consistent with the EPR data is a Δ^{12}-[9,10,11]-allyl radical, an isomer of the pentadienyl radical created by rotation about the 11 bond.[3] The lack of delocalization of the electron spin over the Δ^{12} double bond would explain the negligible hyperfine splitting by the proton at C13 seen in our experiments. However, any rotation about the C11–C12 bond sufficiently large to eliminate the conjugation would lead to significant dipolar coupling from the proton at C12 (vide supra). The proton at C12 has an isotropic hyperfine coupling of less than 0.6 mT; thus we estimate the dihedral angle between the Δ^{12} π orbital and the allylic radical π orbital to be less than 25°. Such a small rotation should allow substantial delocalization of the radical over the second double bond. This contradiction rules out a vinylallyl radical structure for the species we observe, but not necessarily as an intermediate of the lipoxygenase reaction.

Species in which the iron is coordinated to the fatty acid radical are also superficially attractive. The iron would be

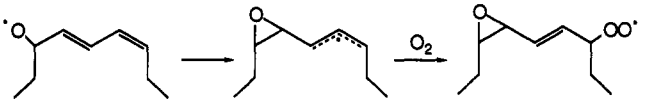
[2] The estimate should use the average of the hyperfine couplings from the *exo* and *endo* protons on C9; the former is usually <0.1 mT greater than the latter. Using only the splitting value we observe (which arises presumably from an *exo* proton) introduces an error of approximately 5% in the spin density at C9 and approximately 2° in the dihedral angle.

[3] On the basis of rotational barriers, the completely unconjugated vinylallyl radical (π orbital of the Δ^{12} double bond orthogonal to that of the [9,10,11]-allyl radical) is about 50 kJ/mol less stable than the isomeric pentadienyl radical (MacInnes & Walton, 1985).

a potent site to which to delocalize the electron spin, rationalizing the unusually small hyperfine splittings observed in the EPR spectra. However, one would predict that a paramagnetic metal ion coordinated to the fatty acid backbone would result in much more dramatic acceleration in spin relaxation of the radical than we observe (Nelson et al., 1990).

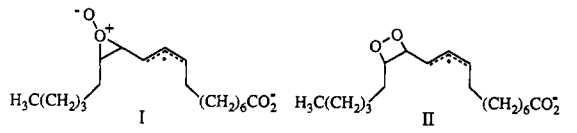
Addition of a substituent across the Δ^{12} double bond is the most reasonable explanation for the lack of hyperfine coupling by the C12 and C13 protons. The most likely covalent modifications involve dioxygen for the following reasons. First, there is a dioxygen dependence to the formation of the allyl radicals from linoleic acid.[4] In the absence of dioxygen, addition of linoleic acid results in reduction of the iron (De Groot et al., 1975b); samples prepared in this way are not purple and evince no allyl radical. On the other hand, the purple color and the allyl radical *are* generated from 13(*S*)-HPOD in the absence of exogenous dioxygen, presumably because the 13(*S*)-HPOD supplies the oxygen in the form of the hydroperoxide. Second, production of the spectroscopically identical radical by the addition of linoleic acid and dioxygen on the one hand, and 13(*S*)-HPOD on the other, implies substitution by oxygen in some form at the C13 end of the radical.

One such oxygen-substituted allyl radical has been detected by EPR spin-trapping studies of the lipoxygenase peroxidase reaction. Cyclization of the alkoxyl radical resulting from homolytic O–O bond cleavage of 13(*S*)-HPOD yields a 12,-13-epoxy-[9,10,11]-allyl radical (Iwahashi et al., 1991; Wilcox & Marnett, 1993). This structure is superficially consistent with the EPR spectra of the allyl radical; it also would be expected to react with dioxygen to yield a 9-peroxyl radical.



Production of these two radicals on the enzyme surface by peroxidase chemistry thus appears to be consistent with the data we present. However, two lines of evidence argue against this interpretation. First, the EPR spectra of the 1-hydroxy-, 1-methoxy-, and 1-*tert*-butoxyallyl radicals show reductions in the hyperfine couplings of the allyl protons of less than 0.1 mT when compared to the unsubstituted allyl radical (Griller et al., 1984). These reductions presumably arise from a small amount of delocalization of the unpaired electron over the (neutral) oxygen atom. One would expect less spin to be delocalized over the oxygen of the 12,13-epoxyallyl radical because the oxygen is one atom farther removed from the radical. Therefore the 12,13-epoxyallyl structure will not rationalize the difference between the observed proton hyperfine couplings and those expected for an alkyl-substituted allyl radical (0.2–0.3 mT, vide supra). Second, the peroxidase chemistry that generates the oxiranylallyl radical requires ferrous lipoxygenase to convert 13(*S*)-HPOD to the alkoxyl radical. The experiments in which the oxiranylallyl radicals were trapped utilized a catalytic system including ferrous lipoxygenase, 13(*S*)-HPOD, and linoleic acid to reduce the ferric lipoxygenase formed by the peroxidase reaction (Iwahashi et al., 1991). In contrast, the experiments that give rise to the purple complex and the allyl radical are performed with ferric lipoxygenase and no reducing agent. In particular, our 13(*S*)-HPOD contains no detectable linoleic acid. Consequently, the conditions of the experiment are not consistent with significant peroxidase chemistry.

[4] R. A. Cowling and M. J. Nelson, unpublished.



Structures **I** and **II** are two possible products of the addition of dioxygen across the Δ^{12} double bond and are analogous to postulated intermediates in the reaction of singlet dioxygen with alkenes (Frimer, 1979).[5] Both structures lead us back to the question of whether an oxygen substituent at C12 will rationalize the smaller than expected hyperfine couplings observed in the EPR spectrum of the allyl radical. In both **I** and **II** the oxygen atom at C12 is substantially more electropositive than that in the oxiranylallyl radical; this could result in greater delocalization of the spin away from C9 and C11. This is testable, however, because such delocalization should result in significant ^{17}O hyperfine splitting in EPR spectra of ^{17}O-enriched samples. Spectra of the allyl radical prepared from 23% [^{17}O]-enriched [per-^{2}H]-13(*S*)-HPOD show no evidence for ^{17}O hyperfine splitting (data not shown); however, simulations suggest that ^{17}O hyperfine coupling of less than about 0.5 mT would not be detectable at such low enrichments. Consequently these data do not rule out an electropositive oxygen atom at C12.

Structure and Origin of the Peroxyl Radical. The peroxyl radical we observe is a 9-peroxyl derivative of linoleic acid, not a 13-peroxyl derivative. There is no evidence in the spectrum of the 12,13-^{2}H-labeled peroxyl radical for 13-peroxyl radical in the samples. The 9-peroxyl and allyl radicals observed in these experiments are in a dioxygen-dependent equilibrium (Nelson et al., 1990). This is sensible in light of the structures determined here; the 9-peroxyl is generated from a [9,10,11]-allyl radical by the readily reversible addition of dioxygen.

Location of the Peroxyl and Allyl Radicals. We showed that the 9-peroxyl radical in purple lipoxygenase solutions is relatively immobilized compared to the 9- and 13-peroxyl radicals generated photochemically in neat linoleic acid (Nelson et al., 1990). Comparison of the magnitude of the hyperfine coupling constant of the C9 proton to other allylic peroxyl radicals supports that conclusion. The magnitude of this dipolar coupling varies from 0.2 to 0.74 mT in a series of alkyl peroxyl radicals in a way that suggests that the dihedral angle between the C–H bond and the half-filled π orbital of the peroxyl radical is controlled by steric interaction between the alkyl moiety and the peroxyl (Bennett & Summers, 1973). For the two allylic peroxyl radicals studied in that series, the isotropic hyperfine splitting observed was 0.45 mT. We estimate the isotropic coupling for the C9 proton of the 9-peroxyl radical to be significantly larger, 0.6 mT, implying constraint of the peroxyl away from its preferred solution conformations. This supports the conclusion that a structure is imposed upon the 9-peroxyl by its binding to the enzyme.

The allyl radical is also conformationally limited, as evidenced by the difference between the hyperfine couplings of the two protons on C8. In a frozen unconstrained sample of the allyl radical one would expect to have a distribution of rotamers about the C8–C9 bond, each of which would have a different magnitude of hyperfine coupling to the two C8 protons. The resulting EPR spectrum would be the sum of the contributions from each of these rotamers and, therefore,

[5] The reaction between singlet oxygen and alkenes to yield allylic hydroperoxides is currently thought to go via a perepoxide (e.g., **I**) or related exciplex intermediate. Dioxetanes are found as products of the reaction between singlet oxygen and particularly electron-rich olefins.

would be poorly resolved. In contrast, the highly resolved EPR spectrum of purple lipoxygenase shows strong coupling to only one of the two C8 protons and is therefore consistent with a single dominant rotamer of the C8–C9 bond. This supports the idea that the allyl radical is also bound to the enzyme.

Both the peroxyl and allyl radicals show unexpectedly rapid spin relaxation as revealed by their high values of $P_{1/2}$ as compared to analogous peroxyl and alkyl radicals formed photochemically in neat samples of linoleic acid (Nelson et al., 1990). This implies the existence of another paramagnet weakly magnetically coupled to the allyl and peroxyl radicals, either another radical or the active-site iron. Considering the low concentration of radicals in the sample, and the likelihood that the radicals observed are bound to the enzyme, the latter is the more reasonable possibility. In general such weak magnetic coupling implies significant distance between the iron and the radicals, on the order of 10 Å.

Relevance to the Mechanism of Lipoxygenase. These data suggest that an allyl radical delocalized over carbons 9–11 of linoleic acid can be generated in the active site of lipoxygenase and will add dioxygen at C9 to form a 9-peroxyl derivative. The 9-peroxyl is unlikely to be on the mechanistic path that leads to 13(*S*)-HPOD; however, it could be the precursor of 9-HPOD. In the absence of steric hindrance to the approach of dioxygen, the 9-HPOD formed in this way would be expected to be racemic.

Even under optimal conditions approximately 5% of the product generated by lipoxygenase 1 from linoleic acid is 9-HPOD (Hamburg & Samuelsson, 1967; Gardner, 1989; Nikolaev et al., 1990). In contrast to 13(*S*)-HPOD, which is made with high stereospecificity by lipoxygenase 1 at pH 9, 9-HPOD is estimated to be produced in a ratio of 70:30 to 50:50 *S*:*R*. Previously discussed sources of 9-HPOD include the following: (1) Reaction between dioxygen and linoleic acid bound "backwards" in the active site. This seems unlikely because the incorrectly bound substrate should be oxygenated by the same mechanism that generates 13(*S*)-HPOD; thus 9-HPOD should be synthesized stereospecifically. (2) Reaction between dioxygen and fatty acid radicals released from the active site in aborted enzymatic reactions. The most likely radical to be released to solution, a pentadienyl radical, should yield all four 9(*R*,*S*)- and 13(*R*,*S*)-HPODs, but significant amounts of 13(*R*)-HPOD are not seen. (3) Reaction at C9 between dioxygen and an activated fatty acid in the lipoxygenase active site via a nonstereospecific mechanism. This third hypothesis proposes that the lipoxygenase mechanism includes an enzyme-bound activated fatty acid that can react with dioxygen either stereospecifically at C13 or racemically at C9. This is consistent with two general types of intermediate structures: (1) an activated fatty acid intermediate that is symmetric about C11 (e.g., a pentadienyl radical) and reacts predominantly at the 13(*pro-S*) position because of steric interference to the approach of dioxygen to the 9(*pro-R*), 9(*pro-S*), and 13(*pro-R*) positions; (2) an asymmetric intermediate that reacts at C13 and C9 by fundamentally different mechanisms, the first inherently stereospecific and the second not.

The observation of an allyl radical in these experiments supports the idea that an asymmetric intermediate could be formed in the active site. In that light, it is attractive to consider a Δ^{12}-[9,10,11]-allyl radical, a rotamer of the pentadienyl radical, because it would be expected to show differences in reactivity toward dioxygen addition at C9 and C13. Further, a Δ^{12}-[9,10,11]-allyl radical intermediate would be consistent with the isomerization of the 9(*E*) substrate to the 9(*Z*) product as well as the observed secondary kinetic isotope effect upon substitution of the vinyl protons: both would arise from the delocalization of the 9-ene. Presumably this rotamer would be stabilized by a protein-imposed rotation about C11–C12 bond. Upon release of the radical from the protein the structure should relax to a pentadienyl radical, consistent with the species trapped in solution after anaerobic reduction of lipoxygenase by linoleic acid.

There are two major questions about the viability of a Δ^{12}-[9,10,11]-allyl radical as an intermediate. First, is there sufficient energy in the reduction of the active-site Fe^{3+} to Fe^{2+} to oxidize linoleic acid to an allyl radical (as opposed to the more stable pentadienyl radical)?[3] There are two oleic acid derivatives, 12-iodooleic acid (Rotenberg et al., 1988) and 12,13-epithiooleic acid (Wright & Nelson, 1992), that are efficient reductants of the iron. One interpretation is that the highly oxidizing active-site Fe^{3+} in lipoxygenase generates an allyl radical from these inhibitors; this reaction would be made irreversible by homolytic cleavage of the C–I or C–S

bond to give the 9,11-diene. Thus, at least when followed by an irreversible reaction, oxidation of the Δ^9 double bond to an allyl radical by lipoxygenase appears to be feasible.

Second, how can 13(*S*)-HPOD be generated from the Δ^{12}-[9,10,11]-allyl radical? The reaction of triplet dioxygen with alkenes is very slow. In contrast, the reaction of singlet dioxygen with alkenes readily yields allylic hydroperoxides via intermediates hypothesized to be similar to structures **I** or **II** above. There is, however, no evidence for the formation of free singlet dioxygen as an intermediate in the lipoxygenase dioxygenase reaction.[6]

A potential route to activation of dioxygen by lipoxygenase is via coordination to the metal. Oxidation of the fatty acid substrate to the Δ^{12}-[9,10,11]-allyl radical would leave the active-site iron in the ferrous oxidation state, potentially able to coordinate dioxygen. Bonding of O_2 to Fe^{2+} is generally described as having two components, a σ bond formed by donation of a lone pair from a rehybridized oxygen atom to the iron, and a π bond formed by back-donation of electrons from the iron into the O_2 π^* orbital (Hall, 1988). The calculated bonding scheme has a dominant contribution from the valence-bond structure Fe^{2+}–O_2 ($S = 0$) (Case et al., 1979; Herman & Loew, 1980; Newton & Hall, 1984; Rohmer, 1986; Yamamoto & Kashigawa, 1989). The unusually high reduction potential of the active-site iron in lipoxygenase, while necessary to oxidize the fatty acid substrate, would destablilize any Fe^{2+}–O_2 complex formed by inhibiting the Fe→O_2 π-back-bonding. That also would decrease the electron density on the terminal oxygen of the complex. This logic suggests that dioxygen would bind only weakly to the intermediate ferrous ion, but the complex formed would contain an electrophilic, diamagnetic dioxygen ligand able to attack the Δ^{12} double bond in a manner analogous to the singlet dioxygen–ene reaction. There is only sparse precedent for metal–dioxygen complexes reacting in a manner analogous to singlet dioxygen (Nishida et al., 1992). There is also no evidence that the Fe^{2+} in native lipoxygenase can bind dioxygen (Feiters et al., 1985),

[6] Free singlet oxygen has been detected in lipoxygenase reactions, but arises from the recombination of peroxyl radicals leaking off the enzyme (Kanofsky & Axelrod, 1986).

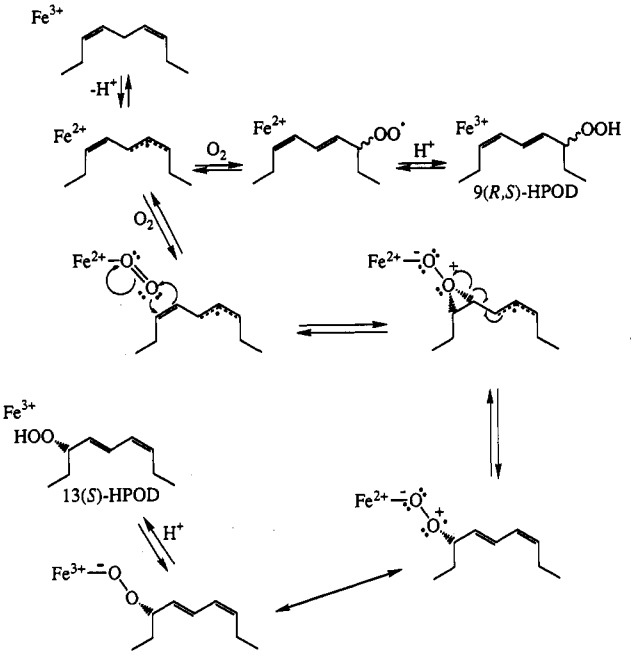


FIGURE 4: A hypothetical mechanism for lipoxygenase involving a vinylallyl radical as an intermediate.

but the Fe^{2+} in the putative intermediate complex could have significantly different reactivity.

A hypothetical mechanism incorporating these ideas is shown in Figure 4. The key features of this mechanism are the following: oxidation of the linoleic acid diene to a Δ^{12}-[9,10,11]-allyl radical by the active-site Fe^{3+}; coordination of the resulting Fe^{2+} by dioxygen to yield an Fe^{2+}–O_2 complex with a diamagnetic dioxygen ligand; electrophilic attack by this complex on the Δ^{12} double bond to yield, e.g., a 12,13-perepoxide; and opening of the perepoxide to the ferric–peroxide complex. The stereospecificity of production of 13(*S*)-HPOD would arise from the position of the iron with respect to the substrate, much as it would in the organometallic mechanism (Corey & Nagata, 1987). Occasional reaction of the Δ^{12}-[9,10,11]-allyl radical with dioxygen would yield the 9-peroxyl, ultimately to generate racemic 9-HPOD. Under this hypothesis, the 12,13-perepoxy-[9,10,11]-allyl radical is the alkyl radical observed in the EPR spectra, and the ferric–peroxide complex gives rise to the purple color of the solutions.

Other interpretations of our data are certainly possible. One is that the radicals we observe are metastable side products of the lipoxygenase reaction and are only relevant to the dioxygenation mechanism in that they confirm the potential for radical reactions in the lipoxygenase active site. For example, structure **I** might arise from rearrangement of 13-peroxyl-9,11-octadecadienoic acid, the peroxyl that leads to 13(*S*)-HPOD in a pentadienyl radical mechanism. It is also possible that lipoxygenase can generate both the Δ^{12}-[9,10,-11]-allyl radical and the Δ^{9}-[11,12,13]-allyl radical. The latter could be the true intermediate; having unpaired spin at C13 it could react with dioxygen and go on to yield 13(*S*)-HPOD. The former, not being on the catalytic path to the dominant product, might be stable enough to be observed, and perhaps react with dioxygen at C9, yielding the 9-peroxyl, and ultimately 9-HPOD. Rapid equilibrium between the two vinylallyl radicals could explain the isomerization of the 9(*E*) substrate to the 9(*Z*) product in that scenario. However, one point against formation of the Δ^{9}-[11,12,13]-allyl radical is that 9,10-epithio-12-octadecenoic acid is not competent to reduce ferric lipoxygenase, in contrast to 12,13-epithiooleic acid (Wright & Nelson, 1992).

Traditionally the requirement for a 1,4-diene in lipoxygenase substrates has been explained by the necessity to form the relatively stable pentadienyl radical as an intermediate. Instead, this mechanism predicts that the role of one of the double bonds of the substrate is to reduce the iron, activating the metal ion to bind and activate dioxygen to, in turn, attack the other double bond. This hypothesis is testable experimentally. Because the proposed intermediate radical is delocalized over only the Δ^{9} double bond of linoleic acid, not Δ^{12}, this mechanism predicts that the secondary kinetic isotope effects reported for the vinyl protons of the substrate (Wiseman, 1989) should be fully expressed upon substitution of only the C9 and C10 protons. It also predicts that the $K_M(O_2)$ for 9-HPOD and 13(*S*)-HPOD formation should be distinct, because the mechanism of dioxygen addition to C13 and C9 is fundamentally different.

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