Enzyme-Bound Pentadienyl and Peroxyl Radicals in Purple Lipoxygenase†

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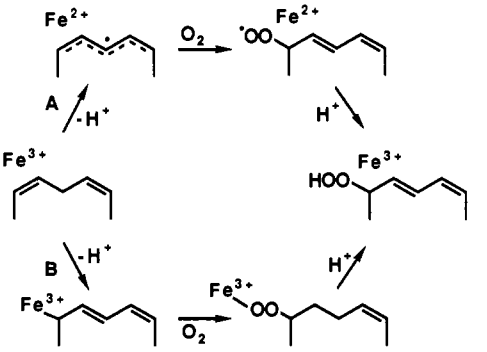
ABSTRACT: Samples of purple lipoxygenase prepared by addition of either 13-hydroperoxy-9,11-octadecadienoic acid or linoleic acid and oxygen to ferric lipoxygenase contain pentadienyl and/or peroxyl radicals. The radicals are identified by the *g* values and hyperfine splitting parameters of natural abundance and isotopically enriched samples. The line shapes of their EPR spectra suggest the radicals are conformationally constrained when compared to spectra of the same radicals generated in frozen linoleic acid. Further, the EPR spectra are unusually difficult to saturate. The radicals are stable in buffered aqueous solution at 4 °C for several minutes. All of this implies that these species are bound to the enzyme, possibly in proximity to the iron. Only peroxyl radical is seen when the purple enzyme is generated with either hydroperoxide or linoleic acid in O_2-saturated solutions. Addition of natural abundance hydroperoxide under ^{17}O-enriched O_2 leads to the ^{17}O-enriched peroxyl radical, while the opposite labeling results in the natural abundance peroxyl radical, demonstrating the exchange of oxygen. Both radicals are detected in samples of purple lipoxygenase prepared with either linoleic acid or hydroperoxide under air. Addition of the hydroperoxide in the absence of oxygen favors the pentadienyl radical. We propose that addition of either linoleic acid or hydroperoxide to ferric lipoxygenase leads to multiple mechanistically connected enzyme complexes, including those with (hydro)peroxide, peroxide, peroxyl radical, pentadienyl radical, and linoleic acid bound. This hypothesis is essentially identical with the proposed radical mechanism of oxygenation of polyunsaturated fatty acids by lipoxygenase.

The mechanism of oxygenation of polyunsaturated fatty acids by lipoxygenases has been a subject of much discussion for well over a decade, with both radical and organometallic species proposed as viable intermediates of the reaction (De-Groot et al., 1975b; Lands, 1984; Feiters et al., 1985; Corey & Nagata, 1987; Corey et al., 1989). Soybean lipoxygenase isozyme 1 is the mechanistic paradigm for these nonheme iron dioxygenases (Veldink & Vliegenthart, 1984); it catalyzes the reaction between linoleic acid (LA)[1] and oxygen to yield 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPO-D). We report the direct observation of two different radical species, both proposed as mechanistic intermediates of the enzyme reaction, stabilized in solutions of "purple" soybean lipoxygenase.

The mechanism shown in Scheme IA, analogous to the known mechanism of autoxidation of linoleic acid (Porter 1986), proposes that the role of the Fe^{3+} ion in the active form of these enzymes is to oxidize the 1,4-diene moiety of the substrate to a pentadienyl radical. In the presence of oxygen this will be in equilibrium with a peroxyl radical that should be competent to reoxidize the Fe^{2+} ion, yielding the peroxide anion. This mechanism is consistent with the following observations: (a) Carbon-centered radicals originating from the substrate are released from the enzyme after addition of linoleic acid to ferric lipoxygenase in the absence of oxygen (De Groot et al., 1973). Under these conditions the iron in the enzyme is reduced. (b) The reduction potential of the iron in ferric lipoxygenase is approximately 0.6 V (versus hydrogen) (Nelson, 1988), much higher than that of most nonheme iron enzymes, as required if the metal ion is to oxidize linoleic acid. (c) 9(*E*),12(*Z*)-Octadecadienoic acid is a slow substrate for

Scheme I



lipoxygenase. The product is, however, 13-hydroperoxy-9-(*Z*),11(*E*)-octadecadienoic acid, demonstrating rotational mobility about the $C_{9,10}$ bond at some point in the reaction (Funk et al., 1987). (d) A broad EPR signal consistent with a peroxyl radical has been detected in a solution of lipoxygenase, linoleic (or arachidonic) acid, and oxygen under flow conditions (Chamulitrat & Mason, 1989). We have reported a well-resolved EPR spectrum with *g* values and ^{17}O hyperfine splitting parameters consistent with an alkyl peroxyl radical in frozen solutions of "purple" lipoxygenase (Nelson & Cowling, 1990); before (d) there had been no published report of direct observation of these radical intermediates.

An alternate mechanism has been suggested (Scheme IB) (Corey & Nagata, 1987; Corey et al., 1989). In this hypothesis the role of the Fe^{3+} ion is to assist in the deprotonation of the diene moiety by coordinating the resulting carbanion. Insertion of dioxygen into the iron–carbon bond followed by protolytic iron–oxygen bond cleavage would yield the product. This mechanism is attractive because it yields the stereospecificity

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

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and regioselectivity of lipoxygenases without resort to a postulate of steric hindrance of the approach of oxygen to one face or end of the intermediate. In this scheme observation (a) above may be rationalized as occurring from slow homolytic iron–carbon bond cleavage that does not compete with oxygen insertion under aerobic conditions and (d) by reaction of that pentadienyl radical with oxygen in solution. However, there is no direct evidence of the critical organometallic intermediate required by this mechanism.

Our observation of an alkyl peroxyl radical, presumed to be 13-dioxy-9,11-octadecadienoic acid, occurred while studying "purple" lipoxygenase prepared by addition of linoleic acid and dioxygen to soybean lipoxygenase isozyme-1 (Nelson & Cowling, 1990). Purple lipoxygenase is an uncharacterized metastable state of lipoxygenase formed by addition of either linoleic acid and oxygen or 13-HPOD to the ferric enzyme (de Groot et al., 1975a). It is distinguished by a band in the visible spectrum at 585 nm with an extinction coefficient in excess of 1300 M^{-1} cm^{-1}. (These solutions are presumed to contain multiple enzyme complexes; so a precise determination of the extinction coefficient is not possible.) The appearance of the purple color is coincident with dramatic changes in the CD spectrum in the 300–450-nm region (Spaapen et al., 1979) and a shift in the relative amounts of $g = 6$ and $g = 4.3$ signals in the EPR spectrum (Slappendel et al., 1983). Consequently it is assumed that the purple color is associated with the metal site, but more definite information is not available. In this report we describe our characterization of two different radicals stabilized in samples of purple lipoxygenase.

Experimental Procedures

Linoleic acid was purchased from Sigma Chemical Co. $^{17}O_2$ (36% enriched) was from Mound Laboratories. 13-HPOD was synthesized by addition of 100 mg of linoleic acid in ethanol to 400 mL of oxygen-saturated buffer (0.05 M borate, pH 8.5) containing 25–50 mg of purified soybean lipoxygenase-1 (Hamburg & Samuelsson, 1967). The reaction was followed spectrophotometrically (λ_{max} = 234 nm for 13-HPOD) to completion; the solution was acidified to pH 4 with acetic acid and extracted with ether. The ether layer was dried with Na_2SO_4 and evaporated to an oil. This was redissolved in a 35:64:1 ethyl acetate–hexane–acetic acid mixture and chromatographed on silica gel. Fractions were analyzed by thin-layer chromatography on silica gel, developed with the same solvent, and visualized with alkaline bromophenol blue. Fractions containing only 13-HPOD were combined and evaporated to dryness. The product was dissolved in ethanol and the concentration was determined from its absorbance at 234 nm [ϵ = 23 600 M^{-1} cm^{-1} (Gibian & Vandenberg, 1987)]. It was divided into aliquots, which were evaporated under nitrogen and stored at −20 °C. Linoleic acid was purified and stored similarly. The same methods were used to prepare and purify $^{17}O_2$-enriched 13-HPOD and [11-^{2}H]13-HPOD, except for the former the buffer was degassed and saturated with 36%-enriched $^{17}O_2$ before addition of linoleic acid or enzyme, and for the latter the substrate was [11,11-$^2H_2$]linoleic acid, synthesized according to the literature (Tucker et al., 1971) and purified by HPLC (silica gel; 1% acetonitrile in hexane).

Soybean lipoxygenase isozyme-1 was purified and assayed as reported previously (Nelson, 1988). The enzyme samples used in these experiments had a specific activity of at least 250 units/mg and were approximately 10 mg/mL protein. Ferrous (as isolated) lipoxygenase was oxidized to ferric by treatment with linoleic acid under air, followed by dialysis against 0.05 M sodium borate pH 9.0. The purple enzyme was generated at 4 °C by addition of either 13-HPOD or linoleic acid to a sample of ferric lipoxygenase in a stoppered cuvette in the thermostated sample holder of a Hewlett-Packard HP8451a spectrophotometer. After each addition of 13-HPOD or linoleic acid a spectrum was obtained. Addition was terminated after the absorption at 585 nm failed to increase upon further addition. The sample was withdrawn from the cuvette with a chilled syringe, transferred to an EPR tube that had been kept on ice, and frozen in liquid nitrogen. Samples were run under particular atmospheres (i.e., Ar, O_2) by evacuating the cuvette and refilling it with the desired atmosphere on a gas train and then adding a solution of enzyme that had been equilibrated against a large excess of the same atmosphere in a serum vial. Samples for EPR study were transferred to stoppered EPR tubes filled with the same atmosphere.

The decomposition of the purple state was followed by preparing purple lipoxygenase as above and taking spectra as the sample was allowed to incubate at 4 °C. Occasionally aliquots were withdrawn and frozen for EPR study.

The pentadienyl radical and peroxyl radical derivatives of linoleic acid were prepared as reported in the literature (Yanez et al., 1987). Linoleic acid (as received, without purification) was degassed, equilibrated with either argon or oxygen, and irradiated with UV radiation filtered above 254 nm for 5 min at 100 K in the cavity of the EPR spectrometer. The sample under oxygen was annealed at 140 K for 10 min, after which it evinced the EPR spectrum of the peroxyl radical. The sample under argon was annealed at 160 K for approximately 1 h, after which it showed the spectrum of the pentadienyl radical.

EPR Spectroscopy. EPR spectra were obtained at temperatures below 80 K by using a Brüker EM200 spectrometer equipped with an Air Products LTR-3 liquid helium cryostat and above 80 K with a Brüker EM300 spectrometer equipped with a liquid nitrogen cryostat. Simulations were performed by using ESRa II (Calleo Scientific Software Publishers) run on an Apple Macintosh II.

Results

Anaerobic treatment of ferric lipoxygenase at 4 °C with purified 13-HPOD at pH 9 induces a new band in the visible spectrum at 585 nm (De Groot et al., 1975a). The low-temperature EPR spectra of such samples reveal a nearly isotropic signal near $g = 2$ with partially resolved hyperfine splitting from three protons (Figure 1A). A good simulation of this spectrum was obtained by using a g value of 2.0026 and hyperfine splitting parameters of 13.6 (1 H) and 12.9 (2 H) G (Figure 1B). Repeating the experiment with [11-^{2}H]13-HPOD leads to the spectrum shown in Figure 2A; the same parameter set simulated this spectrum well (Figure 2B) after changing the 13.6-G ^{1}H splitting to a 2.1-G ^{2}H splitting [$A_0(^2H)/A_0(^1H)$ = 218/1420 = 0.15 (Wertz & Bolton, 1986)]. Using ^{17}O-enriched 13-HPOD, we obtained the same spectrum shown in Figure 1A. In particular, there are no additional splittings or line broadenings observed in that sample. These results suggest that the radical observed in these experiments results from the loss of both atoms of oxygen from 13-HPOD and likely is the pentadienyl radical of linoleic acid.

Generation of the linoleic acid pentadienyl radical in frozen linoleic acid leads to an EPR spectrum (Figure 3) very similar to that obtained for trilinolein (Yanez et al., 1987). The lack of resolution in this spectrum is the result of conformational heterogeneity of the radical in this sample and suggests that the spectrum in Figure 1A arises from the pentadienyl radical in a limited number of conformations. The EPR spectrum of the linoleoyl pentadienyl radical may show evidence of as many

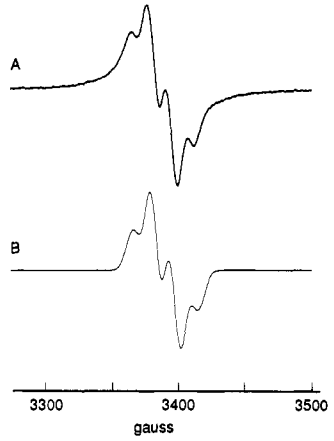


FIGURE 1: EPR spectrum of the radical in samples of purple lipoxygenase formed by addition of 13-HPOD under argon. (A) Sample prepared by addition of 13-HPOD to Fe^{3+} lipoxygenase (approximately 10 mg/mL) at 4 °C until the maximum absorption at 585 nm was attained and then freezing in liquid nitrogen. EPR parameters: microwave frequency, 9.52 GHz; microwave power, 1 mW; modulation amplitude, 3 G; temperature, 30 K. (B) Simulation using $g = 2.0026$; $A = 13.6$ G (1 ^{1}H); $A = 12.9$ G (2 ^{1}H); $A = 4.0$ G (6 ^{1}H).

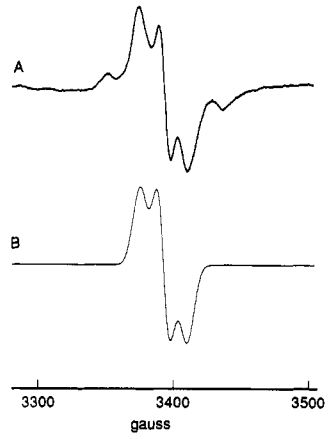


FIGURE 2: EPR spectrum of the radical in samples of purple lipoxygenase formed by addition of [11-^{2}H]13-HPOD under argon. (A) Sample prepared as in Figure 1A, except that [11-^{2}H]13-HPOD was used. The small features on the wings arise from an unidentified impurity seen in only one of four independent samples prepared with the deuterated hydroperoxide. (B) Simulation using the same parameters as in Figure 1B, but with the largest proton splitting replaced by $A = 2.0$ G (1 ^{2}H).

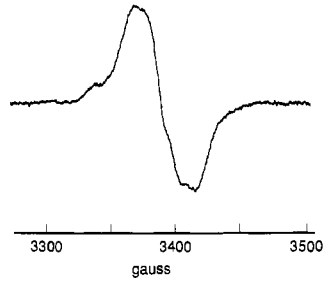


FIGURE 3: EPR spectrum of the genuine pentadienyl radical of linoleic acid in neat linoleic acid. Samples prepared by irradiation of deaerated frozen linoleic acid with UV light for 5 min at 100 K, followed by annealing at 160 K (Yanez et al., 1987). EPR parameters as in Figure 1, except a microwave power of 2 μW.

as seven tightly coupled protons (Bascetta et al., 1982; Yanez et al., 1987), the single protons on C-9, C-11, and C-13 and all four allylic protons on C-8 and C-14. The spectrum in Figure 3 indicates that the pentadienyl radical in frozen linoleic acid is a mixture of rotamers about the $C_{8,9}$ and $C_{13,14}$ bonds. The observed spectrum, then, is the sum of spectra arising from

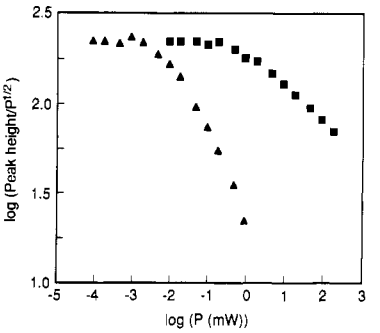


FIGURE 4: Saturation behavior of pentadienyl radicals at 30 K. Radical in frozen linoleic acid (▲). Radical in purple lipoxygenase (■).

individual species with a range of hyperfine splittings from the protons on C-8 and C-14 and therefore is very poorly resolved. The spectrum we observe in purple lipoxygenase (Figure 1A) shows much higher resolution, implying a severely limited set of conformations for the radical. Additionally, we do not observe resolved hyperfine splitting by the four protons on C-8 and C-14 of the linoleoyl radical, implying further restrictions on the set of possible conformations of the radical (vide infra). Minimally, the contrast between the spectra in Figures 1A and 3 suggests that the radical we are observing in the purple lipoxygenase samples is conformationally constrained by being bound to the enzyme.

The presence of the enzyme also has a dramatic effect on the saturation behavior of the pentadienyl radical (Figure 4). The pentadienyl radical in frozen linoleic acid has a half-saturation value of 8 μW at 30 K. However, the radical we observe in the enzyme samples does not show any signs of saturation until above 100 μW at that temperature; we estimated a half-saturation value of approximately 1 mW. This is an unusually high value for an organic radical, especially in comparison to the value seen for the same radical in frozen linoleic acid.

We and others have observed EPR spectra of alkyl peroxyl radicals in samples of lipoxygenase treated with linoleic acid and oxygen (Chamulitrat & Mason, 1989; Nelson & Cowling, 1990). We also generated the peroxyl radical of linoleic acid (presumably a mixture of the 9- and 13-peroxyl fatty acids) photochemically in frozen linoleic acid. Comparison of the EPR spectra of the peroxyl radicals in the presence and absence of enzyme as a function of temperature suggests conformational constraints on the structure of the radical. In Figure 5 are spectra of linoleoyl peroxyl radicals in frozen linoleic acid and in purple lipoxygenase as a function of temperature from 100 to 180 K. The spectra at 100 K are essentially identical with those obtained at 30 K, and those of the peroxyl radical in neat linoleic acid agree well with spectra previously published (Becker et al., 1987). The peroxyl radical in neat linoleic acid is a mixture of both 9- and 13-peroxyl fatty acids; consequently it might be expected to yield a broader spectrum at all temperatures. Note, however, that the spectra of both samples broaden and evince a new feature at about 3350 G as the temperature is raised. This effect results from mobility of the peroxyl radical (Becker et al., 1987) and is more evident in the spectra of samples that do not contain lipoxygenase. This implies conformational constraint imposed upon the peroxyl radical, likely by binding to the enzyme.

The saturation behavior of the peroxyl radical in frozen linoleic acid and in purple lipoxygenase is shown in Figure 6. The half-saturation value for the radical in frozen linoleic acid is 1 mW, while the same radical in the purple lipoxygenase samples has a half-saturation value of approximately 8 mW.

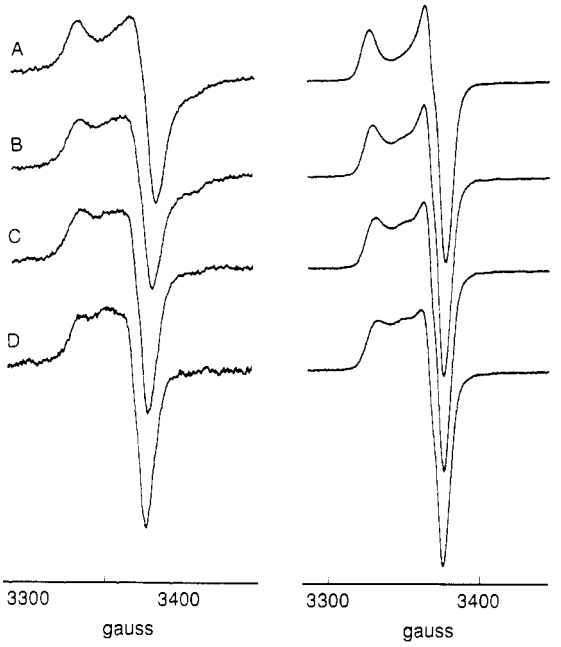


FIGURE 5: EPR spectra of linoleoyl peroxyl radicals as a function of temperature. Left, linoleoyl peroxyl radical generated in oxygen-saturated frozen linoleic acid. Right, peroxyl radical in purple lipoxygenase. EPR parameters as in Figure 1, except a microwave power of 4 mW. Temperature: (A) 100 K; (B) 140 K; (C) 160 K; (D) 180 K.

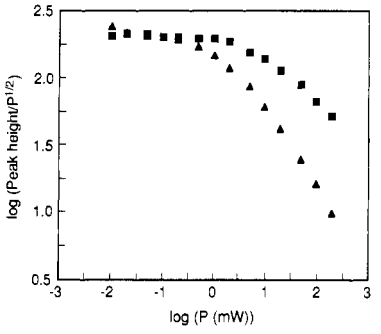
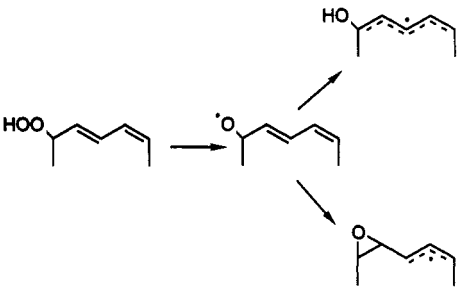


FIGURE 6: Saturation behavior of peroxyl radicals at 30 K. Radical in frozen linoleic acid (▲). Radical in purple lipoxygenase (■).

As in the case of the pentadienyl radical, the presence of the enzyme increases the amount of microwave power required to saturate the EPR transition, although here the effect is not as dramatic because the peroxyl radical is already very difficult to saturate.

The formation of these two radicals appears to be mechanistically related and controlled by the concentration of oxygen. Samples of purple lipoxygenase generated in air by using either 13-HPOD or linoleic acid have EPR spectra arising from a mixture of pentadienyl and peroxyl radicals. Spectra of purple lipoxygenase generated by addition of either 13-HPOD or linoleic acid under an atmosphere of oxygen show predominantly the peroxyl radical. Further, the EPR spectrum of a sample generated by addition of natural abundance 13-HPOD under an atmosphere of ^{17}O-enriched O_2 shows the hyperfine splitting characteristic of ^{17}O enrichment of the peroxyl radical. In the converse experiment, using $^{17}O_2$-enriched 13-HPOD and an atmosphere of natural abundance oxygen, no detectable ^{17}O hyperfine splitting of the peroxyl radical was observed. This demonstrates the exchangeability of the oxygen in these species; according to the known chemistry of peroxyl radicals this occurs via loss of a molecule of dioxygen and generation of an oxygen-free radical species, the pentadienyl radical (Porter, 1986). Finally, in the absence of oxygen, addition of 13-HPOD leads to the generation of the purple color with coincident appearance of the pentadienyl radical. Under these conditions linoleic acid effectively reduces ferric lipoxygenase, but we observe no pentadienyl radical in these samples.

Scheme II



The presence of both of these radicals is correlated with the purple color. The intensities of the 585-nm absorption band and the EPR signals increase in concert when ferric lipoxygenase is titrated with either linoleic acid under oxygen or 13-HPOD under argon. Double integration of the EPR spectra of samples with maximum absorption at 585 nm shows from 0.1 to 0.2 spin per iron of the peroxyl radical in the former and 0.05 to 0.2 spin per iron of the pentadienyl radical in the latter. Samples of purple lipoxygenase left at 4 °C gradually lose their purple color, reverting to the native ferric state. During this process the 585-nm band and the EPR signals decrease in intensity. However, the peroxyl and pentadienyl radicals in samples of purple lipoxygenase treated with 1 equiv of either linoleic acid or 13-HPOD do appear to be stable on the order of minutes at 4 °C. The same radicals in frozen linoleic acid disappear rapidly when the samples are annealed at 190 K in the EPR cavity, presumably because the matrix becomes soft enough to allow reaction with near-neighbor hydrogen atom donors (Yanez et al., 1987). This suggests substantial stabilization of these radicals in the presence of the enzyme, even though they are in aqueous solution. This is precisely what would be expected for mechanistic intermediates in the enzyme active site.

Discussion

The presence of pentadienyl and peroxyl radical intermediates in the oxygenation of unsaturated fatty acids by lipoxygenases has been discussed for over a decade (de Groot et al., 1975b). Recently a broad EPR signal with the g value of an alkyl peroxyl radical was observed in samples of lipoxygenase exposed to fatty acid substrates and oxygen under flow conditions (Chamulitrat & Mason, 1989). We have reported an EPR spectrum with g values and ^{17}O hyperfine splitting parameters of an alkyl peroxyl radical in frozen solutions of purple lipoxygenase prepared by addition of linoleic acid under oxygen (Nelson & Cowling, 1990). In this paper we report further characterization of that radical as well as the observation of a different organic radical in samples of purple lipoxygenase prepared by addition of 13-HPOD to ferric lipoxygenase in the absence of oxygen.

This new signal represents a derivative of 13-HPOD itself, as shown by the effect of ^2H substitution at the 11-position of 13-HPOD on the EPR spectrum of the radical. Failure to observe any effect on the spectrum of ^{17}O-enrichment in 13-HPOD almost rules out the presence of an oxygen nucleus in the radical system. In particular, it eliminates the possibility that the radical we see is the 13-hydroxylinoleoyl radical (Scheme II), conceivably the result of isomerization of a 13-oxyl radical produced by the lipoxygenase peroxidase activity. It also mitigates against an oxiranylallyl radical (Scheme II);

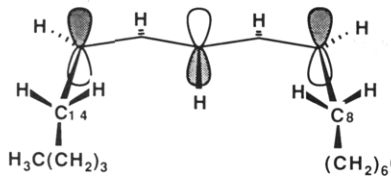


FIGURE 7: One of the possible configurations of the pentadienyl radical in purple lipoxygenase. This assumes a planar diene structure. Structures with small deformations from planarity are also consistent with the data (see text).

however, we cannot be definitive on this point in the absence of a suitable model.

We are left with the conclusion that this spectrum most likely represents the pentadienyl radical derivative of linoleic acid, identical with the proposed intermediate of the oxygenation reaction. This assignment is based on comparisons to EPR spectra of the analogous radical studied in neat solutions of linoleic acid (Bascetta et al., 1982), as well as those of pentadienyl radical itself (Griller et al., 1979; Sustmann & Schmidt, 1979). The EPR spectrum of the *E,E* form of pentadienyl radical[2] shows hyperfine splittings of 11.58 (1 H), 10.40 (2 H), 9.62 (2 H), and 3.32 (2 H) G. On the basis of molecular orbital calculations (Hinchliffe & Cobb, 1974), the largest coupling may be assigned to the proton on C-3 (numbering for pentadiene), the next four to the exo and endo protons on C-1 and C-5, and the smallest two couplings to the protons on C-2 and C-4. This is consistent with the unpaired electron being in a nonbonding molecular orbital with highest density on C-1, C-3, and C-5 and nodes at C-2 and C-4, as expected for a planar, delocalized π-electronic system.

The EPR spectrum of the *E,E* form of the linoleoyl pentadienyl radical obtained in liquid solution at 290 K shows a similar hyperfine splitting pattern: 11.1 G (1 H), 9.8 G (2 H), 7.8 G (4 H), and 3.15 G (2 H). These splittings are assigned to the proton on C-11, the two protons on C-9 and C-13, the four protons on C-8 and C-14, and the two protons on C-10 and C-12, respectively (for the numbering system, see Figure 7). The splittings from the protons on C-8 and C-14 are a function of the angle of the C–H bond with respect to the π-orbital that contains the unpaired electron: $A_H = B\rho \cos^2 \theta$, where B is approximately 45 G and ρ is the electron spin density at the carbon (Sevilla & Holroyd, Furimsky et al., 1980; Koppenol & Butler, 1985). Assuming free rotation about the $C_{8,9}$ and $C_{13,14}$ bonds in these solution samples, $B\rho$ = 15.6 G. By comparison to these published results, we assign the 13.6-G splitting in the spectra of purple lipoxygenase to the proton on C-11 and the 12.9-G splittings to the protons on C-9 and C-13 and conclude that we are unable to resolve the splittings from the other six protons in these frozen samples.

This assignment assumes that the pentadienyl radical we are observing is planar and the unpaired electron is delocalized over both of the double bonds of the fatty acid. However, it is conceivable that the enzyme might force a twist in the $C_{10,11}$ bond, diminishing the ability of the electron to delocalize over the $C_{9,10}$ double bond. Because the unpaired spin would be concentrated on fewer atoms, the proton hyperfine splittings would be expected to be somewhat larger in such a conformation. In the extreme, a 90° rotation about the $C_{10,11}$ bond would effectively yield an allyl radical with a vinyl substituent. The EPR spectrum of an allyl radical would be expected to show hyperfine splittings from the C-11 and C-13 protons of 12.9–14.8 G (Davies et al., 1981; Bascetta et al., 1982). The magnitude of the splittings we observe in the EPR spectra of purple lipoxygenase are indeed somewhat larger than expected for a planar pentadienyl radical, possibly implying some rotation about the $C_{10,11}$ bond. A full 90° rotation would imply the proton on C-10 would contribute a hyperfine splitting larger than any we see [$B\rho$ is approximately 25.8 G for the methylene protons adjacent to an allyl radical (Bascetta et al., 1982)]. Assigning one of the 12.9-G splittings to that proton yields a maximum rotation of about 45° in this model, while assigning one of the unresolvable splittings gives a rotation of less than 35°.

It is interesting to note that the regioselectivity of the oxygenation reaction could be partially explained if the enzyme did indeed distort the intermediate radical away from a planar structure. This would increase the spin density on C-13 at the expense of C-9, making the former carbon more attractive to attack by oxygen. The energetic cost of a small rotation about the $C_{10,11}$ bond cannot be estimated precisely, but the activation energy barrier to the 180° rotation that converts pentadienyl radical from *E,E* to *E,Z* in the gas phase is 11.7 ± 0.5 kcal/mol (MacInnes & Walton, 1985). Further, any rotation away from planarity will increase the energy required to generate the pentadienyl radical; for example, the C–H bond dissociation energies of propene and 1,4-pentadiene differ by 6–12 kcal/mol (Golden & Bensen, 1964; Egger & Cocks, 1973; Rossi & Golden, 1979).

Determination of extent of deviation from planarity in the pentadienyl radical bound to the enzyme with require assigning each of the proton hyperfine splittings in the EPR spectrum, by use of specifically deuterated fatty acid hydroperoxides—their synthesis is in progress. Again, on thermodynamic and spectroscopic grounds any such deviation must be relatively small. In addition, the observation that the enzyme produces the 9(*Z*) product from 9(*E*),11(*Z*)-octadecadienoic acid demonstrates that the bond order of the $C_{9,10}$ bond is reduced at some stage in the mechanism to allow for the isomerization (Funk et al., 1987), implying substantial delocalization of the radical into that double bond.

Comparison of the EPR spectrum of the genuine linoleoyl pentadienyl radical, obtained at 30 K in neat linoleic acid, with the spectrum of the radical in purple lipoxygenase shows substantial increase in resolution in the presence of the enzyme. The poor resolution seen in the spectrum of the radical in frozen linoleic acid suggests multiple conformations about the $C_{8,9}$ and $C_{13,14}$ bonds of the radical, leading to contributions from species with a range of hyperfine splittings from the protons on C-8 and C-9. The EPR spectrum observed for the radical in frozen linoleic acid potentially comprises contributions from individual species with many values for θ; thus A_H may range from 0 to 15.6 G and the summation is the poorly resolved spectrum of Figure 3. The much higher resolution seen in purple lipoxygenase implies most of those conformations are eliminated in the presence of the enzyme.

In fact, the conformational constraint on the pentadienyl radical in purple lipoxygenase must be extremely severe because we do not observe resolved hyperfine splitting by these protons at all. The hyperfine splitting from the C-8 and C-14 protons of a planar pentadienyl radical would be unresolvably small only if θ for each C–H bond were approximately 60° (A_H = 3.9 G). Note that rotamers with $\theta > 60°$ (and thus $A_H < 3.9$ G) for one C–H bond would have $\theta < 60°$ (and $A_H > 3.9$ G) for the other C–H bond on the same carbon. Therefore, θ must be within about 10 deg of 60° for each of the allylic C–H bonds if all four of these protons are to yield

[2] The 11(*E*) double bond in the enzymatic product, 13-HPOD, implies that the intermediate is either the (*E*),(*E*)- or (*E*),(*Z*)-pentadienyl radical. For simplicity we have restricted ourselves to the *E,E* form, but the discussion that follows applies to both.

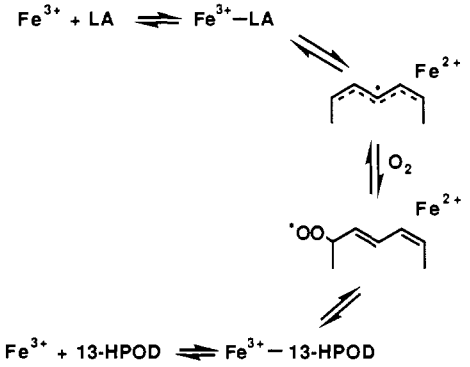


FIGURE 8: Hypothetical mechanism for the formation of purple lipoxygenase.

unresolved hyperfine splitting. This is consistent with the radical being constrained into a few conformations in these samples, all of which have the $C_{7,8}$ and $C_{14,15}$ bonds nearly perpendicular to the plane of the pentadienyl radical, and enables us to make a reasonably detailed prediction for the structure of the bound intermediate from carbons 7–15 (Figure 7), over half the length of the substrate. Note that the same argument for conformational restraint holds in the case of the twisted diene model, as we would expect a mixture of rotamers about the $C_{13,14}$ bond to diminish the resolution of the spectrum of the radical.

Both the peroxyl and pentadienyl radicals seen in samples of purple lipoxygenase appear to be bound to the enzyme in or near the active site. First, both radicals appear to be under motional and/or conformational constraint, implying they are bound to the enzyme. Second, the saturation behavior of both radicals is perturbed strongly by the presence of the enzyme, revealing an increase in the rate of relaxation of each radical. Similar anomalously rapid relaxation of tyrosyl radicals in ribonucleotide reductase and photosystem II have been taken as evidence for weak coupling to a nearby paramagnetic species that facilitates the relaxation of the organic radical (Sahlin et al., 1987; Innes & Brudvig, 1989). It is plausible to assume the same phenomenon in these purple lipoxygenase samples; however, the only other paramagnetic species known in this enzyme is the iron, which is paramagnetic in both the ferric and ferrous states. We take this as circumstantial evidence for binding of both radicals relatively near the iron and thus in or near the active site of the enzyme. Finally, these radicals appear to be substantially stabilized in these samples, as compared to the same radicals in frozen linoleic acid, just what one would expect of mechanistic intermediates bound in the active site.

The question arises, how is it that we have stabilized putative mechanistic intermediates in these samples? We propose the reaction scheme shown in Figure 8. This is merely the hypothetical radical mechanism of fatty acid oxygenation by lipoxygenase, in which all the steps are explicitly written as equilibria. The key point is that we generate purple lipoxygenase in concentrated enzyme samples, generally about 0.1 mM. We hypothesize that this effectively drives the system into the fully complexed state and allows the intermediates to approach their thermodynamic equilibrium concentrations. Thus addition of 0.1 mM 13-HPOD to 0.1 mM ferric lipoxygenase results in stoichiometric formation of the enzyme–13-HPOD complex, which then may partition among the various bound states shown in Figure 8. In the absence of oxygen, the system is driven into states with the pentadienyl radical or linoleic acid bound; the only radical species is the pentadienyl radical, as observed. Under an atmosphere of oxygen, the system is driven into states in which the peroxyl radical, hydroperoxide, or peroxide is bound; in that case the only radical is the peroxyl radical, as observed. Under air, a mixture of pentadienyl and peroxyl radicals is obtained. This hypothesis also accounts for the observation that under ^{17}O-enriched O_2, natural abundance 13-HPOD yields $^{17}O_2$-enriched peroxyl radical, and under natural abundance O_2, $^{17}O_2$-enriched 13-HPOD yields no detectable ^{17}O-enriched peroxyl radical. The exchange of O_2 in the peroxyl radical in these samples is most plausibly explained by oxidation of the hydroperoxide to the peroxyl in which the dioxygen is in facile equilibrium with the solution, via the pentadienyl radical (Porter, 1986). From the data, however, we cannot determine whether the exchange is taking place on the enzyme rather than in solution, although the latter would seem unlikely as it requires the radicals to survive dissociation from the enzyme.

This scheme implicitly assumes that the oxygenation reaction catalyzed by lipoxygenase is driven thermodynamically by release of the hydroperoxide product. Our observations suggest that at least the radical steps of the mechanism are close to isoenergetic, as both radical intermediates may be observed simultaneously under the appropriate conditions. Further, the large primary isotope effect observed upon substitution of ^2H for ^1H at the 11-position of linoleic acid [k_H/k_D = 8.7 (Egmond et al., 1973)] suggests that ΔG for the hydrogen abstraction step is "within several kilocalories per mole of zero" (Miller & Klinman, 1983; Bell, 1973). Finally, although the relative amounts of the radicals we observe are small, they represent a significant fraction of the amount of enzyme present; thus, the equilibrium constants between the states containing radicals and those containing closed-shell intermediates are also within about an order of magnitude of 1. These data, then, suggest that all of the reactions that occur in the active site of lipoxygenase between binding and substrate and dissociation of product are nearly isoenergetic.

Our hypothesis makes a number of testable predictions. First, the EPR spectra should reveal a stoichiometric decrease in the amount of Fe^{3+} (as it is reduced to Fe^{2+}) upon the appearance of these radicals. We do see a decrease in the intensity of the signals from Fe^{3+} in these samples, but quantitative interpretation is complicated by the difficulty in integrating the iron spectra, which are in excess of 2000 G wide. Indeed, we require particularly accurate integrations because the signals from the radicals account for less than 0.2 spin/mol. These experiments are in progress. Second, quantifying the relative proportion of peroxyl and pentadienyl radical in samples titrated with linoleic acid or 13-HPOD in the presence of varying concentrations of oxygen should allow determination of the effective equilibrium constant for the reaction of the pentadienyl radical with oxygen. This, too, is in progress. Finally, we predict that the purple color arises from one or more of these intermediate states. We know that none of the radical-bound states is purple, because addition of a trace of ethanol [known to bind near the active site (Slappendel et al., 1982)] eliminates the radicals. The ethanol-treated samples are, however, still purple. We also observe that the maximum absorption at 585 nm achievable by addition of linoleic acid is a function of oxygen concentration (Cowling and Nelson, unpublished results). This implies a role for oxygen in the production or stability of the purple species; the latter has already been suggested (Slappendel et al., 1983). From all of these results we suggest the purple color is associated with the state or states with bound alkyl (hydro)peroxide. Possibly the color results from a ligand-to-metal charge-transfer absorption band in a complex produced by

substitution of one of the "native" protein ligands by either the alkyl peroxide or a different protein ligand, possibly either cysteine or tyrosine. This may be an important mechanistic point because the existence of a ferric–peroxide complex would imply that the reduction of the peroxyl radical to generate the product is an inner-sphere process. On the other hand, substitution of a tyrosine or cysteine at the iron presumably would decrease its reduction potential and alter the possible roles of the metal in the reaction.

There is another plausible mechanism to explain our results: oxidation of the iron from Fe^{2+} to Fe^{3+} in those states that contain the pentadienyl or peroxyl radical would remove the most immediate source of electrons for quenching the radicals, thus stabilizing them. The most likely oxidant is 13-HPOD, which is present (either by addition or enzymatic synthesis) in concentrations in excess of those of the radical species. Comparison of the amount of radical formed with the change in concentration of Fe^{3+}, by EPR spin integration (vide supra), will address this point directly. In the end, however, this explanation of our results also strongly supports the hypothetical radical mechanism of oxygenation.

In summary: we have obtained evidence for pentadienyl and peroxyl radicals stabilized in samples of purple lipoxygenase. These species appear to be connected mechanistically via oxygen, in accord with the known chemistry of such radicals. Both radicals appear to be bound to the enzyme and possibly are weakly magnetically coupled to the active-site iron. This is the strongest evidence yet to support the proposed radical mechanism of lipoxygenase (Scheme 1A). We cannot, however, rule out all of the steps of the alternate mechanism (Scheme 1B) because the oxidation of linoleic acid to the pentadienyl radical could proceed via either an outer- or inner-sphere process, the latter being homolytic Fe–C cleavage of an as-yet-undetected intermediate organometallic complex.

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