Photolysis of "Purple" Lipoxygenase: Implications for the Structure of the Chromophore†

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ABSTRACT: Treatment of soybean lipoxygenase isozyme 1 with its substrates, linoleic acid and oxygen, or product, 13(*S*)-hydroperoxy-9,11(*Z*,*E*)-octadecadienoic acid (13-HPOD), leads to the appearance of a purple color. Although the structure of the chromophore has not been determined, we present strong evidence that it is an Fe^{3+}−OOR complex between the enzyme and 13-HPOD. Irradiation of frozen purple solutions of lipoxygenase causes the reversible production of a radical, shown by the effects of ^{2}H and ^{17}O enrichment on its EPR spectrum to be derived from 13-HPOD. The action spectrum of the photolysis reaction corresponds to the visible spectrum of the purple species, strongly implying that the purple chromophore contains 13-HPOD (or a product thereof) as part of its structure. Concomitant with the production of this radical there is a decrease in the intensity of an EPR signal corresponding to enzyme-bound Fe^{3+} and characteristic of the purple species. Taken together, these observations support the suggestion that the purple species is a complex between ferric lipoxygenase and 13-HPOD, likely the ferric peroxide.

Lipoxygenases are non-heme iron dioxygenases that catalyze the incorporation of dioxygen into polyunsaturated fatty acids. Soybean lipoxygenase isozyme 1, the most thoroughly studied of this class, uses linoleic acid *in vitro* to produce 13(*S*)-hydroperoxy-9,11-octadecadienoic acid (13-HPOD)[1] (Gardner, 1989; Siedow, 1991).

Two distinct chemical mechanisms for lipoxygenases have been proposed. In one mechanism the non-heme ferric ion in the active enzyme is proposed to oxidize the 1,4-diene unit of the substrate to a neutral radical that can react directly with dioxygen (de Groot et al., 1975b). The products, a fatty acid peroxyl radical and the active-site ferrous ion, would react to form the hydroperoxide anion, possibly via a ferric−fatty acid peroxide complex. In the second mechanism the iron forms a σ-organometallic complex with the deprotonated substrate, and dioxygen inserts into the Fe−C bond, yielding a ferric−fatty acid peroxide complex (Corey & Nagata, 1987). A ferric−13-HPOD complex is a viable intermediate in both of these mechanistic hypotheses.

"Purple" lipoxygenase is generated in the laboratory by the addition of substrates (linoleic acid and dioxygen) or product (13-HPOD) to solutions of the active, ferric form of soybean lipoxygenase at reduced temperature (de Groot et al., 1975a). Visible spectra of these solutions have a broad absorption at approximately 585 nm ($\epsilon > 1000\ M^{-1}\ cm^{-1}$) not present in spectra of solutions of the native, ferric enzyme (Spaapen et al., 1979). There is evidence that this purple species is an intermediate of the dioxygenase reaction catalyzed by lipoxygenase. First, when formed using linoleic acid and dioxygen, the purple color appears at the same rate as product (Egmond et al., 1977). Second, the only alkyl hydroperoxide that generates the purple color in soybean lipoxygenase isozyme 1 is 13-HPOD, the dominant enzyme product. In particular, the 9-hydroperoxide isomer does not generate the purple species from this isozyme (de Groot et al., 1975a). This suggests that the purple species may be an enzyme−product complex. There are examples of synthetic Fe^{3+}−OOR complexes that have absorption bands above 500 nm (Nishida & Akamatsu, 1991; Zang et al., 1993); in one case it has been shown by resonance Raman that this band has peroxide-to-iron charge-transfer character (Zang et al., 1993).

Spectroscopic evidence suggests that purple lipoxygenase does contain an altered form of the active-site iron. EPR spectra of the frozen purple solutions show a mixture of iron species, including those corresponding to the native, ferric enzyme as well as a new signal ($g_{1,2,3} \approx 4.3$) arising from Fe^{3+} in a rhombic electronic environment (de Groot et al., 1975a; Slappendel et al., 1981). The intensity of this EPR signal correlates with the intensity of the 585-nm absorption band (Slappendel et al., 1983), suggesting they arise from the same structure. EPR spectra also reveal the presence of enzyme-bound radicals in these samples (Nelson et al., 1990).

We hypothesize that the purple solutions are a mixture of species, some of which are intermediates of the dioxygenase reaction. Elucidating the structure of the purple species is a critical test of this hypothesis. Resonance Raman spectroscopy is a powerful probe of the structure of such chromophores; however, we have been unable to obtain a resonance Raman spectrum of purple lipoxygenase, despite exhaustive efforts. A potential explanation of this is that the purple species is photolabile and decomposes in the excitation beam. In this paper we show that purple lipoxy-

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[1] Abbreviation: 13-HPOD, 13(*S*)-hydroperoxy-9,11-octadecadienoic acid.

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genase is indeed photolabile, reversibly photolyzing to yield a fatty acid radical derived from 13-HPOD.

MATERIALS AND METHODS

Perdeuteriolinoleic acid was obtained from Cambridge Isotope Laboratories and 9(*S*)-hydroperoxy-10,12-octadecadienoic acid from Biomol Research Laboratories. The specifically deuterated fatty acids and fatty acid hydroperoxides, [per-^{2}H]-13-HPOD, and 40%-enriched [$^{17}O_2$][per-^{2}H]-13-HPOD were synthesized as previously described (Nelson et al., 1994). Soybean lipoxygenase isozyme 1 was purified, and samples of purple lipoxygenase were generated as previously described (Nelson, 1988; Nelson et al., 1994).

EPR Spectroscopy. EPR spectra were obtained using a Bruker ER-200D spectrometer with an Oxford Instruments ESR900 cryostat. To obtain spectra of the radicals, the conditions were *T*, 30 K; modulation amplitude, 0.6 mT; microwave power, 2 mW; microwave frequency, 9.42 GHz; and magnetic field sweep rate, 0.17 mT/s. To obtain spectra of the ferric ions, the conditions were *T*, 5 K; modulation amplitude, 1 mT; microwave power, 10 mW; microwave frequency, 9.42 GHz; and magnetic field sweep rate, 1.2 mT/s. Spectra were integrated using a Bruker computer and quantified by comparison to a standard sample of Cu(EDTA) run at the same temperature. Integrals of the $g = 4.3$ signals were corrected for g_{av} (Aasa & Vänngård, 1975) and for zero-field splitting by assuming that *D*, the zero-field splitting parameter, $\ll kT$ (Chasteen et al., 1993), so the number of spins in the middle Kramers doublet giving rise to the signal is approximately one-third of the total number of spins.

Irradiation of EPR Samples. Frozen samples of purple lipoxygenase in EPR tubes were placed in the cryostat of the EPR spectrometer, and spectra were taken. With the temperature set to 30 K the sample was illuminated using a Cermax LX300UV xenon illuminator with a total output of approximately 0.05 W/nm through the range from 400 to 800 nm. The light was filtered through 6 cm of circulating tap water to filter IR radiation and, unless stated otherwise, through various Corian high-pass filters. The sample temperature increased less than 5 K during the irradiation. After irradiation the sample temperature was reestablished, and EPR spectra were obtained. In some cases the samples were removed from the cryostat and allowed to incubate at either 77 K (liquid nitrogen) or approximately 200 K (dry ice/acetone) in the dark. After incubation the samples were reintroduced to the cryostat, and new spectra were obtained.

RESULTS

Treatment of ferric lipoxygenase (after dialysis to remove the fatty acid products of the oxidation) with 13-HPOD generates a purple chromophore, with maximum absorption at 585 nm occurring after addition of approximately 1 equiv of 13-HPOD; K_D for the formation of the purple color with 13-HPOD has been estimated to be 41 μM at 4 °C (Wang et al., 1993). In contrast, treatment of 0.05 mM ferric lipoxygenase (generated by treatment with 13-HPOD and dialysis) with 30 equiv of the 9(*S*)-hydroperoxide did not yield any absorbance at 585 nm, leading to a minimum value for K_D of 15 mM (assuming $<10\%$ of the enzyme reacted with the hydroperoxide). Thus, in agreement with previously published data (de Groot et al., 1975a), soybean lipoxygenase-1 exhibits substantial preference for 13-HPOD, its dominant product, in the formation of the purple species.

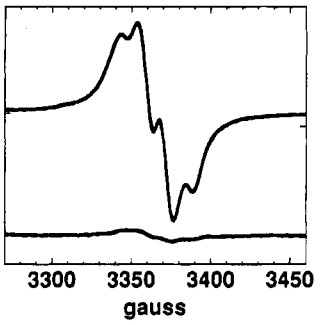

FIGURE 1: EPR spectrum of photoirradiated purple lipoxygenase. Ferric lipoxygenase was treated with 10 equiv of 13-HPOD, frozen, and irradiated at 30 K for 10 min as stated in the text. EPR conditions are also in the text. Curves: top, after irradiation; bottom, before irradiation.

Samples of purple lipoxygenase prepared with an excess of natural abundance 13-HPOD under anoxic conditions show an EPR feature at $g = 2$ with partially resolved hyperfine splittings (Nelson et al., 1990). We have shown that these spectra arise from fatty acid allyl radicals bound to the enzyme. UV−visible irradiation of such samples leads to a much more intense EPR signal in the $g = 2$ region of the spectrum. This new signal shows no resolution of hyperfine splittings, indicating that it results from photolysis of 13-HPOD in the frozen aqueous solution, rather than on the enzyme (Nelson et al., 1994). The magnitude of the through-space superhyperfine coupling between the unpaired spin and a proton on the fatty acid backbone depends on $\cos^2 \theta$, where θ is the angle between the orbital that contains the radical and the C−H bond. The frozen solution contains 13-HPOD in many different conformations, so the radicals generated have a distribution of values of θ and therefore a distribution of superhyperfine couplings. The resulting EPR spectrum is the sum of the spectra of the individual species, with a wide variety of hyperfine splittings, and thus is broad and poorly resolved.

In contrast, when the sample is irradiated using a 450-nm high-pass filter, there is a smaller but still dramatic increase in intensity of the EPR spectrum in the $g = 2$ region (Figure 1). The spectrum generated by irradiation under these conditions does show partial resolution of splitting by three strongly coupled protons. The resolution apparent in this spectrum is evidence that the radicals generated are in a very limited set of conformations, probably because they are bound to the enzyme (Nelson et al., 1990). This is supported by the observation that increasing the amount of fatty acid hydroperoxide beyond approximately 2 equiv does not result in an increase in the amount of radical formed during the irradiation. That is the same amount of 13-HPOD required to generate the maximum intensity of the 585-nm absorption band in these experiments. Although further addition of 13-HPOD increases the amount of fatty acid hydroperoxide in solution, it does not increase the amount of radical formed by irradiation. This supports the idea that the more highly resolved spectrum represents radicals formed on the enzyme rather in solution.

Irradiation of a sample of purple lipoxygenase formed using [per-^{2}H]-13-HPOD gave a singlet EPR spectrum, showing that all of the strongly coupled protons on the radical come from the fatty acid backbone. Samples prepared with either [8,8-^{2}H]- or [9,10-^{2}H]-13-HPOD evinced triplet EPR signals, while samples prepared with [12,13-^{2}H]-13-HPOD gave a spectrum identical to that in Figure 1 after photolysis.

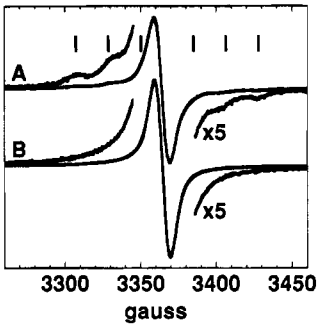

FIGURE 2: EPR spectra of photoirradiated purple lipoxygenase (A) prepared with 40%-enriched [$^{17}O_2$][per-^{2}H]-13-HPOD and (B) prepared with [per-^{2}H]-13-HPOD. The vertical lines in (A) indicate the positions of the features assuming 21.6 G ^{17}O hyperfine splitting.

This demonstrates that the radical generated by the photolysis is a [9,10,11]-allyl radical—similar to the radical in unphotolyzed samples of purple lipoxygenase (Nelson et al., 1994).[2] When purple lipoxygenase was prepared with 40%-enriched [^{17}O][per-^{2}H]-13-HPOD and photolyzed, spectrum A in Figure 2 was obtained. Features arising from ^{17}O hyperfine splitting are apparent in this spectrum, demonstrating the presence of at least one oxygen nucleus from 13-HPOD in this radical.

Samples of purple lipoxygenase prepared with either 13-HPOD or linoleic acid under oxygen have prephotolysis EPR spectra arising from 9-peroxyl fatty acid radicals generated by reaction of dioxygen with the allyl radical (Nelson & Cowling, 1990; Nelson et al., 1994). Irradiation of these samples again leads to an increase in the EPR spectrum, but the new signal generated is that of the [9,10,11]-allyl radical, not the peroxyl radical. The allyl radical produced by irradiation of samples prepared with 40%-enriched [^{17}O][per-^{2}H]-13-HPOD under natural abundance dioxygen showed the same ^{17}O splittings (after subtraction of the peroxyl radical signal of the unphotolyzed sample) seen in the samples prepared anoxically. In contrast, the spectra of irradiated samples prepared using [per-^{2}H]-13-HPOD under 40%-enriched $^{17}O_2$ did not show ^{17}O splittings. Thus the oxygen nucleus in the fatty acid allyl radical comes from 13-HPOD not dioxygen.

The action spectrum of this photolysis reaction was obtained using a series of high-pass filters from 450 to 800 nm (Figure 3). Little or no photoproduction of the radical was seen when filters of 600 nm or longer wavelength were used. With filters of 550 nm or shorter wavelength, the increase in the radical signal observed after irradiation paralleled the integral of the absorption spectrum of the purple species. This suggests that the radical production occurs as the result of absorption by the 585-nm chromophore.

The time course of the photolysis at approximately 30 K is shown in Figure 4. A sample of purple lipoxygenase was prepared with a 10-fold excess of 13-HPOD and irradiated using the 450-nm high-pass filter. After 50 min the photolysis was essentially complete, and the sample no longer appeared purple. Integration of the radical signal obtained after 50 min of photolysis showed approximately 0.24 spin/

[2] No care was taken to exclude light during the preparation of purple lipoxygenase samples studied in the previous work. No significant reproducible difference in the intensity of the radical signals was noticed between samples prepared in the dark and under normal laboratory illumination.

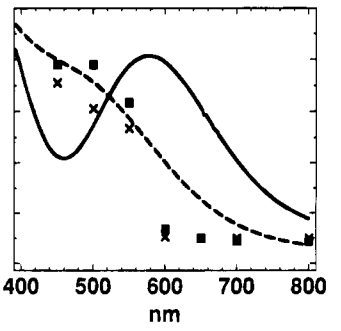

FIGURE 3: Action spectrum of the photolysis of purple lipoxygenase: (—) absorption spectrum of purple lipoxygenase; (- - -) integrated absorption spectrum of purple lipoxygenase. Samples were prepared with 10 equiv of 13-HPOD and irradiated for 10 min. Symbols: relative amount of radical in samples prepared under O_2 (×) and under Ar (■).

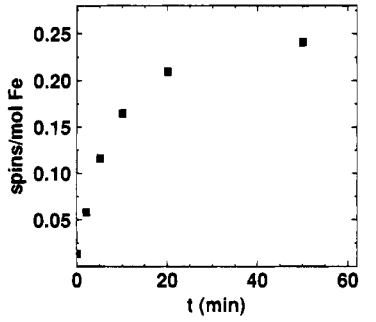

FIGURE 4: Time course of the appearance of the radical during photoirradiation.

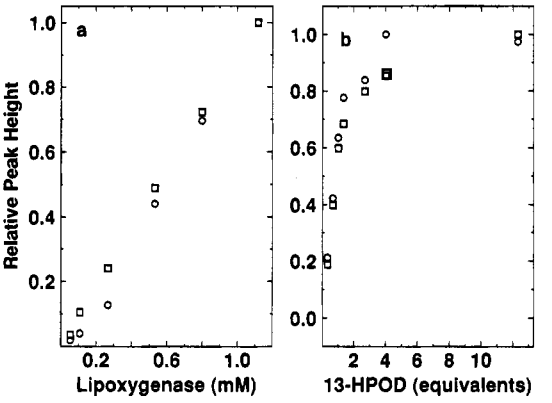

FIGURE 5: Dependence of the $g = 4.3$ EPR signal (○) and the 582-nm absorption band (□) of purple lipoxygenase on the enzyme and 13-HPOD concentration. (a) Ten equivalents of 13-HPOD was added to ferric lipoxygenase. (b) Various concentrations of 13-HPOD were added to ferric lipoxygenase (10 mg/mL).

iron in the sample. After the sample was thawed in ice—water, the purple color returned, presumably as the result of exchange of 13-HPOD from solution with the radical in the active site.

Formation of purple lipoxygenase by addition of either 13-HPOD or linoleic acid and oxygen to ferric lipoxygenase also leads to the appearance of a new EPR signal at $g = 4.3$, arising from high-spin Fe^{3+} in a nearly rhombic electronic environment (de Groot et al., 1975a). A correlation between the intensity of this EPR signal and the purple color has been demonstrated (Slappendel et al., 1983). Both of these features are linear functions of enzyme concentration between 0 and 100 mg/mL ferric lipoxygenase (Figure 5a) in samples prepared with a 10-fold excess of 13-HPOD. Alternatively, titration of 10 mg/mL (0.1 mM) ferric lipoxygenase with 13-HPOD leads to a linear increase in the

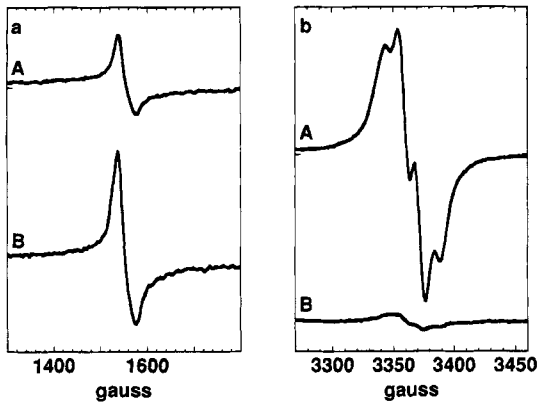

FIGURE 6: Effect of photoirradiation on the rhombic iron (a) and radical (b) EPR signals in purple lipoxygenase (A) after 10 min of irradiation and (B) before irradiation.

intensity of both the $g = 4.3$ EPR signal and the 585-nm absorption band (Figure 5b). This is circumstantial evidence that the two spectroscopic features arise from the same enzyme chromophore.

Figure 6 shows the change in intensity of the $g = 4.3$ and $g = 2$ EPR signals resulting from 10 min of irradiation. The $g = 4.3$ signal is substantially smaller after the irradiation, while the signal arising from the radical product of the photolysis is much stronger. The ferric ions that disappear from the $g = 4.3$ signal do not reappear elsewhere in the spectrum, and it is assumed that they have been reduced to the ferrous state. The ratio of the spins gained in the $g = 2$ signal by photolysis to the spins lost from the $g = 4.3$ signal was 1.1 ± 0.4 (21 samples). This suggests that the radical arises from the photolysis of the iron species associated with the $g = 4.3$ EPR signal.

The reversibility of the photolysis was examined with samples of purple lipoxygenase prepared with approximately 0.8 equiv of 13-HPOD. In these samples there should be little 13-HPOD remaining in solution, preventing regeneration of the purple species by exchange of 13-HPOD from solution into the active site. These samples were irradiated and allowed to stand in the dark for up to 5 h. Spectra of samples that were kept at 30 K were identical to those taken immediately after irradiation, both in the $g = 4.3$ region and in the $g = 2$ region. Samples kept at 77 K showed some conversion of the allyl radical to the 9-peroxyl radical; nonetheless, the total concentration of radical dropped to about 50% of that seen immediately after irradiation, and the intensity of the $g = 4.3$ signal showed a corresponding increase. Considering the temperature of the experiment and the excess of enzyme over 13-HPOD present, this must be the result of a reaction of the radical bound to the enzyme. Finally, samples maintained at 200 K also showed some conversion of the allyl radical to the peroxyl radical, with an overall decrease in the amount of radical present but no corresponding increase in the $g = 4.3$ signal.

DISCUSSION

The structure of purple lipoxygenase is a subject of substantial curiosity as well as potential relevance to the mechanism of the enzymatic reaction. The purple species appears to be a mechanistically competent complex between ferric lipoxygenase and its major product, 13-HPOD. We and others have hypothesized that the 585-nm absorption that gives rise to the purple color is a ligand-to-metal charge-transfer band in a coordination complex between the ferric ion in the active site and 13-HPOD (Zang et al., 1993; Nelson et al., 1994). In support of this, solutions of purple lipoxygenase contain a form of Fe^{3+} substantially different than that found in native ferric lipoxygenase (de Groot et al., 1975a; Slappendel et al., 1981). This notion is also supported by model chemistry: a synthetic ferric−alkyl peroxide complex has been shown to have a peroxide-to-iron charge-transfer band at 515 nm (Zang et al., 1993).

Unfortunately, resonance Raman spectroscopy has not yielded any information about the structure of the chromophore in purple lipoxygenase. We have shown here that the purple species is photolabile, and excitation at wavelengths shorter than 600 nm in the Raman experiment likely leads to decomposition of the sample. We have studied the products of this photodecomposition reaction, hoping to find a clue to the structure of the purple species.

Irradiation of purple lipoxygenase samples at 30 K with visible light between 400 and 600 nm leads specifically to production of enzyme-bound fatty acid radicals. The action spectrum of the reaction corresponds to the spectrum of the purple chromophore. The radicals generated by the photolysis contain nonexchangeable oxygen and deuterium nuclei from the 13-HPOD used to generate the purple enzyme. These two observations make it virtually certain that the fatty acid hydroperoxide is structurally associated with the purple chromophore.

The loss of a form of ferric ion assigned to the purple chromophore concomitant with the production of the radical suggests that the ferric ion in the enzyme active site is also structurally associated with the purple chromophore. This supports the assignment of the 585-nm absorption band of the purple species to a 13-HPOD peroxide-to-iron charge transfer. This suggests strongly that the structure of the purple species is an Fe^{3+}−13-HPOD complex.

The decrease in the EPR signal arising from this ferric ion is not accompanied by an increase in any other EPR signal ascribable to Fe^{3+}; consequently, one product of the irradiation appears to be Fe^{2+} and the other a fatty acid radical. We suggest that irradiation of purple lipoxygenase leads to homolysis of the iron−oxygen bond in the excited state of the transition. Note that the photolysis occurs only on the high-energy side of the optical transition, where the excited state has sufficient vibrational energy for the bond to break.

The products of the dissociation, Fe^{2+} and fatty acid peroxyl radical, would be expected to be short-lived; in fact, we have not observed the peroxyl directly. We suggest that the fatty acid allyl radical we do observe results from rearrangement of the peroxyl radical in the active site of the enzyme. Note that release of either the fatty acid peroxyl or the peroxide from the enzyme is unlikely in these samples at cryogenic temperatures.

The photolysis reaction is slowly reversible. At 77 K after 5 h, only about 50% of the allyl radical had disappeared with production of the $g = 4.3$ iron species. On the other hand, incubation at 200 K for 5 h led to a similar decrease in the amount of radical observed with no increase in the amount of $g = 4.3$ iron signal. At 77 K the frozen sample matrix is fairly firm, and the degree of motional freedom of the radical is substantially limited. The only chemistry available is reconversion to the purple species. At 200 K, though, the matrix is much "softer", and the radical has substantially more motional freedom. This is consistent with

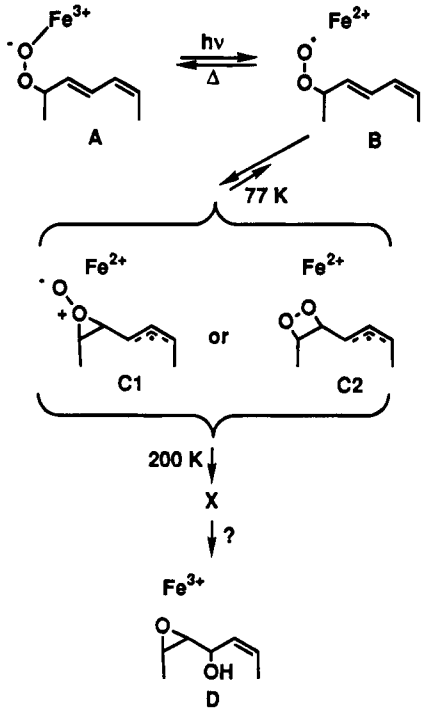

FIGURE 7: Plausible mechanism for the photolysis reaction and the subsequent radical rearrangements.

the temperature dependence of the motion of, e.g., linoleyl peroxyl radical in neat linoleic acid (Becker et al., 1987). In that case the mobility of the fatty acid radical increased dramatically between 77 and 193 K. A similar temperature dependence of the motion of the radical in our system would allow it to react with itself or with the protein at 200 K but not at 77 K.

We propose the reaction sequence shown in Figure 7. This is based on several hypotheses: (1) The purple species is a ferric−13-HPOD complex (structure A) responsible for a peroxide-to-iron charge-transfer absorption at 585 nm. The evidence for this is discussed above. (2) Illumination into this peroxide-to-iron charge-transfer band results in homolytic cleavage of the Fe−OOR bond, leaving Fe^{2+} and the 13-peroxyl radical.[3] We expect this reaction to be readily reversible on thermodynamic grounds. (3) This allylic peroxyl radical can rearrange to an allyl radical with either a dioxetane (C2) or perepoxide (C1) substituent—we propose that this is the radical we observe.[4] (4) At 77 K this radical slowly rearranges back to the peroxyl which rapidly re-forms the purple species, but at 200 K it reacts irreversibly with itself or the protein. Structure D in Figure 7 has been shown to be a product of the reaction of soybean lipoxygenase with 13-HPOD (Corey & Mehrotra, 1983). Similar though structures C1 and D might be, there is no evidence that D results from the allyl radical we observe. The direct decomposition of the purple species in these experiments to, e.g., structure D is ruled out by the observation that the $g = 4.3$ signal is stable for 5 h at 200 K in unirradiated samples, even when the purple species is prepared with substoichiometric amounts of 13-HPOD.

There is no precedent for us to estimate whether the ^{17}O nucleus at C-12 in C1 or C2 would give as large a hyperfine splitting as we observe. We do note that dioxetanes are known to decompose with C−C bond cleavage (Kearns, 1971); decomposition of C2 in this way would generate the 12-oxo-[9,10,11]-allyl radical, in which the spin would be delocalized over the oxygen nucleus leading to large ^{17}O hyperfine coupling. We expect the C−C cleavage reaction to be irreversible, thus eliminating the possibility of re-forming the purple species from the 12-oxo-[9,10,11]-allyl radical. This is inconsistent with the observed reversibility of the photolysis reaction at 77 K.

In this work we have demonstrated that irradiation into the 585-nm absorption band of purple lipoxygenase leads to a reversible decrease in the amount of an Fe^{3+} species associated with the purple complex and the production of a radical derived from 13-HPOD. This is strong evidence in support of the hypothesis that the chromophore in purple lipoxygenase is a ferric−13-HPOD complex, possibly the penultimate intermediate of the dioxygenase reaction.

[3] We see no evidence for the 13-peroxyl radical, but its presumed location near the high-spin ferrous ion should lead to strong relaxation broadening. In addition, the nearly stoichiometric conversion of the $g = 4.3$ iron species into the allyl radical suggests any intermediates exist at very low concentrations.

[4] Only cyclization of the peroxyl to C12 of the fatty acid could give rise to a radical consistent with the EPR spectra. Such a cyclization could proceed via either the terminal or the inner oxygen of the peroxyl because there is significant spin density on both atoms (Sevilla et al., 1990).

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