Action of Soybean Lipoxygenase 1 on 12-Iodo-*cis*-9-octadecenoic Acid and 12-Bromo-*cis*-9-octadecenoic Acid†

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ABSTRACT: The ferric form of soybean lipoxygenase catalyzes an elimination reaction on 12-iodo-*cis*-9-octadecenoic acid (12-IODE) to produce iodide ions and 9,11-octadecadienoic acid (9,11-ODA). If excess 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD) is present, the reaction proceeds until about one-half of the racemic 12-IODE is consumed; in the absence of excess 13-HPOD, the reaction stops after about three turnovers. Ferric lipoxygenase also catalyzes the conversion of 12-bromo-*cis*-9-octadecenoic acid (12-BrODE) to 9,11-ODA at a rate that is less than 25% of that observed with 12-IODE. These elimination reactions cannot be detected with ferrous lipoxygenase or with lipoxygenase that has been inactivated by 5,8,11,14-eicosatetraynoic acid. In the case of 12-IODE, elimination is accompanied by a loss of enzymatic activity; at pH 9.0, about 10 iodide ions are produced per molecule of enzyme inactivated. No inactivation can be detected with 12-BrODE. Ascorbate and hydroxylamine, which can act as free-radical traps, block the inactivation by 12-IODE but do not inhibit the elimination reaction. When the enzyme is inactivated by [1-^{14}C]-12-IODE at pH 9.0, the amount of radioactivity that is covalently bound to the protein is less than 30% of that expected for 1:1 incorporation. Considerable radioactivity from [1-^{14}C]-12-IODE becomes noncovalently associated with the protein; most of this radioactivity can be removed by extraction with ethyl acetate, and thin-layer chromatography of the ethyl acetate extracts indicates that they contain a complex mixture of radioactive substances that are more polar than 12-IODE and 9,11-ODA. These materials may be formed by a free-radical pathway, since the noncovalent incorporation of radioactivity is greatly reduced by the presence of ascorbate. The noncovalent incorporation of these materials does not appear to be the cause of inactivation, since extraction of most of the radioactivity with ethyl acetate does not restore catalytic activity, and since noncovalent incorporation occurs to about the same extent at pH 7.6 as at pH 9.0, even though inactivation is much less at pH 7.6. The results suggest that the main pathway for inactivation of lipoxygenase by 12-IODE involves the enzymatic conversion of 12-IODE to a radical (or some other reactive species that can be trapped by ascorbate and hydroxylamine), which irreversibly modifies the enzyme without covalent attachment of the carbon skeleton of the inactivator to the protein.

Lipoxygenases are non-heme iron proteins that catalyze the oxygenation of *cis,cis*-1,4-dienes to produce conjugated diene hydroperoxides. For example, soybean lipoxygenase 1 catalyzes the conversion of linoleic acid to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD).[1] Lipoxygenases are widespread in plants and have been implicated in a variety of physiological processes (Gardner, 1991; Seidow, 1991; Maccarrone et al., 1994). In mammals, lipoxygenases are involved in the conversion of arachidonic acid to leukotrienes, lipoxins, and other inflammatory mediators (Yamamoto, 1992; Serhan, 1994; Ford-Hutchinson et al., 1994). Inhibitors of human neutrophil arachidonate 5-lipoxygenase, which is involved in leukotriene synthesis, are of potential value in the treatment of inflammation and anaphylaxis (Lewis et al., 1990; Batt et al., 1992), and inhibitors of other lipoxygenases would be useful in clarifying their physiological functions.

Purified soybean lipoxygenase 1 is ESR-silent, and the iron has been shown to be in the ferrous state (Slappendel, 1982; Cheesbrough & Axelrod, 1983; Whittaker & Solomon, 1988; Funk et al., 1990). Treatment of the enzyme with 1 equiv of its product, 13-HPOD, results in rapid conversion to an ESR-active ferric form (DeGroot et al., 1975; Pistorius et al., 1976). Similar results have recently been obtained with recombinant arachidonate 5-lipoxygenase from human neutrophils (Chasteen et al., 1993) and with arachidonate 15-lipoxygenase from rabbit reticulocytes (Carroll et al., 1993). The crystal structure of the ferrous form of soybean lipoxygenase 1 has recently been reported (Boyington et al., 1993; Minor et al., 1993).

There is considerable evidence that catalysis by soybean lipoxygenase 1 is initiated by reaction of the ferric form of the enzyme with linoleic acid to generate an intermediate, which reacts with oxygen. The nature of the intermediate is uncertain; possible candidates include a pentadienyl radical

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[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 12-IODE, 12-iodo-*cis*-9-octadecenoic acid; *trans*-12-IODE, 12-iodo-*trans*-9-octadecenoic acid; 12-BrODE, 12-bromo-*cis*-9-octadecenoic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ETYA, 5,8,11,14-eicosatetraynoic acid; UV, ultraviolet; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ESR, electron spin resonance.

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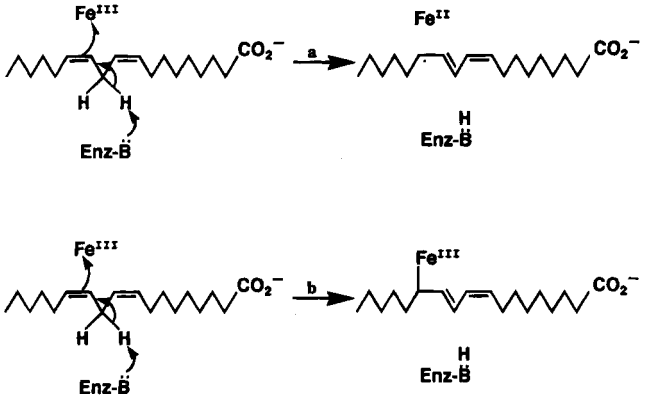
Scheme 1



bound to the ferrous enzyme (DeGroot et al., 1975), as in Scheme 1a, an organoiron species (Corey & Nagata, 1987), as in Scheme 1b, and a Δ^{12}-[9,10,11]-allyl radical bound to the ferrous enzyme (Nelson et al., 1994). Ferric lipoxygenase has been shown to be fully active in the absence of lipid hydroperoxide under single-turnover conditions (Wiseman et al., 1988). Kinetic evidence suggests that ferrous lipoxygenase cannot process linoleic acid until the enzyme has been oxidized to the ferric form by 13-HPOD or other oxidants (Schilstra et al., 1994), although this view has been challenged (Wang et al., 1993).

As a possible approach to elucidating the mechanism by which lipoxygenase catalyzes the cleavage of the C(11)−H bond in linoleic acid, we have been investigating the action of lipoxygenase on substrate analogues in which the 12,13 double bond has been replaced by a leaving group at C(12):

12-IODE, X=I
12-BrODE, X=Br

These studies led to the unexpected discovery that 12-iodo-*cis*-9-octadecenoic acid (12-IODE) is a time-dependent, irreversible inactivator of soybean lipoxygenase (Rotenberg et al., 1988). No inactivation was observed with 12-BrODE, 12-iodooctadecanoic acid, or 9,11-octadecadienoic acid (9,11-ODA), which would be formed by the elimination of HI from 12-IODE. Inactivation by 12-IODE requires 13-HPOD; this result implies that inactivation is specific for the ferric form of the enzyme. In this paper, we report the results of a detailed study of the action of ferric lipoxygenase on 12-IODE and 12-BrODE.

EXPERIMENTAL PROCEDURES

Materials. Lipoxygenase 1 was purified from locally obtained soybeans by the method of Axelrod (1981). Preparations were >90% homogeneous by SDS−PAGE and had specific activities ranging from 175 to 200 units/mg. Protein concentrations are based on M_r 94 000 (Shibata et al., 1987) and $A_{280nm}^{0.1\%}$ = 1.6 (Petersson et al., 1987). Lipoxygenase was assayed as described previously (Rotenberg et al., 1988) on a Beckman DU-40 spectrophotometer interfaced with an IBM PC for data analysis. Linoleic acid was obtained from Sigma and converted enzymatically to 13-HPOD (Gibian & Galaway, 1976). Methyl ricinoleate was prepared from castor oil by the method of Swern and Jordan (1952). 12-IODE, *trans*-12-IODE, and 12-BrODE were synthesized as previously reported (Rotenberg et al., 1988). Glutathione peroxidase (from bovine erythrocytes), glutathione, and dithiothreitol were obtained from Sigma, and L-ascorbic acid was obtained from Aldrich.

Synthesis of [1-^{14}-C]-12-IODE. Methyl 12-hydroxy-*cis*-9-octadecenoate (methyl ricinoleate) was converted to [1-^{14}C]-12-hydroxy-*cis*-9-octadecenoic acid ([1-^{14}C]ricinoleic acid) by the general procedure described previously (Campbell & Clapp, 1989) using K^{14}CN (New England Nuclear) as the source of isotope. Diazomethane was used to convert the labeled ricinoleic acid to its methyl ester, which was converted to [1-^{14}C]-12-IODE by the procedure described for unlabeled material (Rotenberg et al., 1988). The final product was purified by HPLC in system A (see below) and was determined to have a specific radioactivity of 0.44 μCi/μmol.

9,11-Octadecadienoic Acid. This material was synthesized by an elimination reaction on methyl 12-[(methylsulfonyl)-oxy]-*cis*-9-octadecenoate followed by hydrolysis of the methyl ester, as reported previously (Gunstone & Said, 1971; Rotenberg et al., 1988). The product was an approximately 3:1 mixture of two isomers with identical mass spectra. These isomers could be cleanly separated by GC and partially separated by HPLC (Figure 3A). A pure sample of the major isomer was obtained by collecting the early portion of the major HPLC peak, and a 500 MHz NMR spectrum of this material gave vinyl couplings consistent with one *cis* double bond (J = 11 Hz) and one *trans* double bond (J = 15 Hz). By assuming that the *cis* double bond in the starting material is retained in the product, the stereochemistry of the major isomer is 9-*cis*,11-*trans* and the minor isomer is probably 9-*cis*,11-*cis*.

Iodide Determination. Iodide ion was detected by an Orion Model 94-53 iodide electrode used with an Orion Model 90-01 reference electrode and an Orion 701A Ionalyzer. The system was calibrated at 20 °C using sodium iodide in 50 mM borate (pH 9.0) to obtain a plot of electrode potential vs log iodide concentration. Measurements were carried out in a total volume of 0.5 mL in a micro sample dish (Orion No. 920014) that was mounted in a Plexiglas holder constructed so as to allow water from a constant temperature bath (20 °C) to circulate under the dish. Reactions were initiated by the addition of 13-HPOD (in 2.0 μL of ethanol) followed by 12-IODE (in 2.0 μL of ethanol) to a solution of lipoxygenase in 50 mM borate (pH 9.0) that had been thermally equilibrated to 20 °C in a 1.5-mL Eppendorf tube immersed in a water bath; after the solution was shaken, it was transferred to the micro sample dish.

Analysis of Organic Products. Reactions (total volume = 0.5−2.0 mL) were carried out in centrifuge tubes; the quantities given below are per milliliter of reaction mixture. After the appropriate incubation period, the reaction mixture was treated with 12.5 μL of 10 mM glutathione, 5 μL of 10 mM EDTA, and 1 unit of glutathione peroxidase in order to convert excess 13-HPOD to 13-HOD. Three minutes later the reaction mixture was acidified with 10 μL of 1 M HCl and then extracted with three 1-mL portions of ethyl acetate. Centrifugation was necessary to separate the layers. The combined ethyl acetate extracts were concentrated to dryness, and the residue was dissolved in 100 μL of ethanol for

analysis by HPLC (injection volume = 25 μL) in system A or B. In experiments with [1-^{14}C]-12-IODE, it was shown that the ethyl acetate extractions removed 90−95% of the radioactivity.

In some experiments, 500-μL aliquots of the reaction mixture were periodically withdrawn and quenched by addition to an equal volume of ice-cold acetonitrile/H_2O/H_3PO_4 (7.5:3.0:0.12, v:v). Aliquots (500 μL) of the resulting mixtures were analyzed by HPLC using system B.

High-Performance Liquid Chromatography. HPLC was carried out on a Beckman system (two 114 M pumps, a 421 controller, and a 210A injector) using an Alltech 250 × 4.5 mm Adsorbosphere C18 (5 μm) column. System A refers to isocratic elution at 1.0 mL/min with acetonitrile/H_2O (85:15, v:v). System B consisted of 5 min of isocratic elution with acetonitrile/H_2O/acetic acid (50:50:0.1), followed by a 10-min linear gradient to acetonitrile/H_2O/acetic acid (85:15:0.1) and 25 min of isocratic elution with the latter solvent mixture; the flow rate was 1.0 mL/min throughout. UV-absorbing materials were detected with an ISCO V4 UV−visible detector. Radioactive materials were detected with a Radiomatic Flo-One/Beta detector with a 1-mL liquid cell, using Packard Flo-Scint II scintillation fluid pumped at 2.0 mL/min.

Inactivation by [1-^{14}C]-12-IODE. An 8.8 μM solution of lipoxygenase in 990 μL of 50 mM borate (pH 9.0) was assayed twice (specific activity = 188) and then treated with 2.9 μL of 17 mM 13-HPOD in ethanol followed by 6.4 μL of 17 mM [1-^{14}C]-12-IODE (0.44 μCi/μmol) in ethanol. Aliquots (2.0 μL) were assayed periodically for enzymatic activity. After 20 min, the reaction mixture was applied to a 22 × 1.4 cm column of Sephadex G-50 made up in 20 mM Tris Cl (pH 8.0). The column was eluted with the same buffer at 0.5 mL/min at room temperature, and the effluent was collected in 2-mL fractions. Protein concentrations were determined by measuring A_{280}, and 100-μL aliquots were counted for radioactivity. Most of the protein eluted in fractions 7 (0.21 mg/mL) and 8 (0.11 mg/mL), which coincided with a peak of radioactivity (8.5 × 10^3 dpm/mg in fraction 7 and 8.3 × 10^3 dpm/mg in fraction 8). Fraction 7 was extracted with three 2-mL portions of ethyl acetate with centrifuging to separate the layers. Most of the counts were extracted into the ethyl acetate. Following this treatment, the aqueous layer was found to contain 0.15 mg/mL protein and 670 dpm/mg. One milliliter of this layer was treated with 670 mg of solid guanidine hydrochloride and dialyzed overnight against 100 mL of 7 M guanidine hydrochloride. This treatment reduced the protein concentration to 0.09 mg/mL and the radioactivity to 350 dpm/mg.

RESULTS

Iodide Formation from 12-IODE. Experiments with an iodide-specific electrode have demonstrated that inactivation by 12-IODE is accompanied by the release of iodide ions. When 50 μM 12-IODE was incubated with 4.6 μM lipoxygenase and 50 μM 13-HPOD in 50 mM borate (pH 9.0), about 55% of the initial activity was lost over about 10 min, and then inactivation ceased (Figure 1A). During the period when inactivation was taking place, a surge of iodide formation occurred (Figure 1B), and then iodide production slowed to essentially the same rate as that for a control that contained no enzyme. The magnitude of the surge was 24

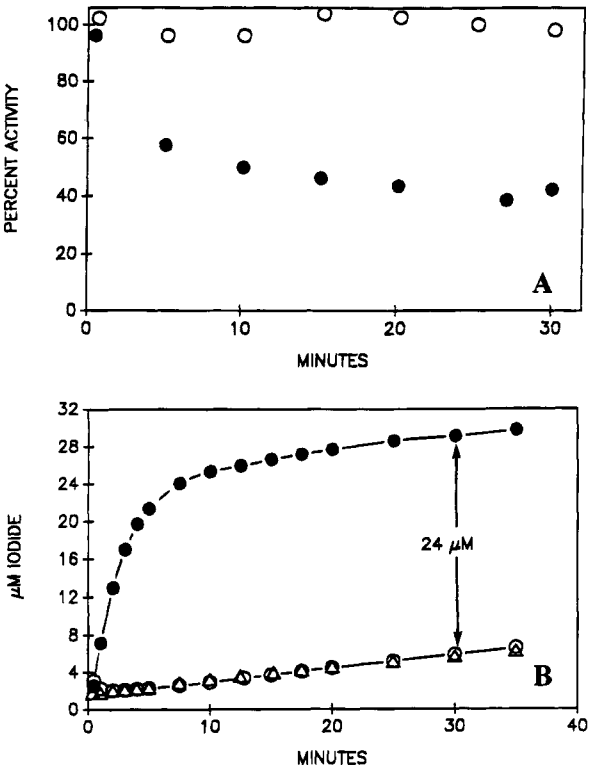


FIGURE 1: Loss of lipoxygenase activity (A) and release of iodide ions (B) from 12-IODE when 13-HPOD is present in excess. Lipoxygenase (4.6 μM, specific activity = 195) was incubated at 20 °C with 12-IODE (50 μM) and 13-HPOD (50 μM) in 50 mM borate (pH 9.0) containing 0.8% (v:v) ethanol (●). The reaction was initiated by the addition of 12-IODE, and aliquots (2.0 μL) were removed periodically and added to 2.5 mL of assay mixture to determine enzymatic activity (panel A). The iodide concentration was determined as described under Experimental Procedures (panel B). Two other experiments were carried out identically except that 13-HPOD was omitted (○) or lipoxygenase was omitted (△).

± 2 μM (mean ± SDEV of three experiments), which implies that one enantiomer of the racemic 12-IODE was processed by the enzyme to produce I^-. If this is correct, then the same enantiomer must also be responsible for inactivation, since when iodide formation ceased, inactivation also ceased. If 13-HPOD was omitted, no inactivation occurred, and iodide production was reduced to the same level as that of the control that contained no enzyme. These results imply that ferrous lipoxygenase is not inactivated by 12-IODE and does not catalyze iodide formation from 12-IODE. The rate of iodide production in the absence of enzyme did not depend on the presence of 13-HPOD (data not shown) and is therefore attributable to nonenzymatic hydrolysis of 12-IODE. The slow iodide production that follows the surge is presumably due to hydrolysis of the enantiomer that is not processed by the enzyme. The magnitude of the surge and the percentage of activity lost are consistent with the formation of approximately 10 iodide ions per molecule of enzyme inactivated.

We have previously shown that *trans*-12-IODE is a much less effective inactivator than the *cis* isomer (Rotenberg et al., 1988). When an experiment identical to that in Figure 1 was carried out with *trans*-12-IODE, iodide formation was greatly reduced. After 20 min, the yield of iodide in the presence of enzyme was only about 4 μM higher than that from a control lacking enzyme. Thus, the *cis* stereochemistry is important for iodide formation as well as for inactivation.

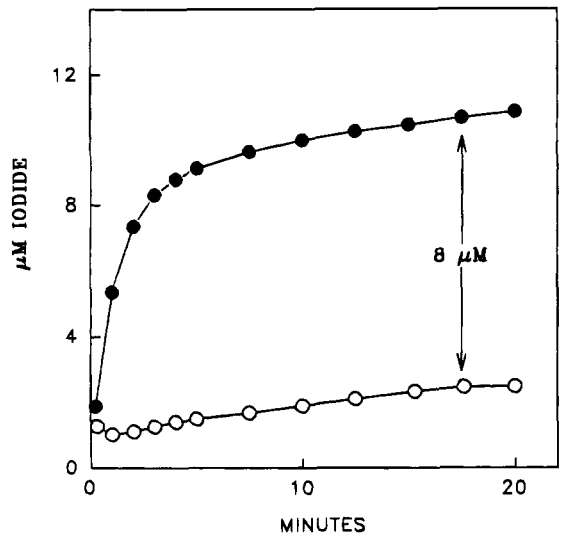


FIGURE 2: Release of iodide ions from 12-IODE in the presence of ferric lipoxygenase without excess 13-HPOD. Lipoxygenase (4.6 μM) in 50 mM borate (pH 9.0) was treated with 13-HPOD (final concn = 2.5 μM) followed at time zero by 12-IODE (final concn = 50 μM) (●). The release of iodide ions at 20 °C was monitored as described under Experimental Procedures. A control was carried out identically except that lipoxygenase was omitted (○).

When 50 μM 12-IODE was incubated with 4.6 μM lipoxygenase in the presence of 2.5 μM 13-HPOD (Figure 2) rather than 50 μM 13-HPOD (Figure 1), the yield of iodide was reduced to 8.3 ± 0.5 μM. In the experiment in Figure 2, the enzyme and 13-HPOD were preincubated for several seconds prior to the addition of 12-IODE; during this preincubation, the 13-HPOD should oxidize 2.5 μM lipoxygenase to the ferric form.[2] (A control experiment demonstrated that the time course of iodide formation was not affected by extending the preincubation period to 10 min.) Thus, when 12-IODE was added to the reaction mixture, it was exposed to 2.5 μM ferric lipoxygenase with no excess 13-HPOD. The results imply that ferric lipoxygenase is capable of catalyzing about 3 turnovers of iodide release from 12-IODE in the absence of excess 13-HPOD. A possible interpretation of this result is that ferric lipoxygenase becomes reduced as the elimination proceeds and is completely reduced after about 3 equiv of iodide has been formed. All subsequent experiments were carried out in the presence of excess 13-HPOD.

Formation of 9,11-Octadecadienoic Acid from 12-IODE. Elimination of hydrogen iodide from 12-IODE would produce 9,11-octadecadienoic acid (9,11-ODA), which absorbs near 230 nm due to the conjugated diene chromophore. We therefore looked for the formation of this material using HPLC with UV detection at 234 nm. Lipoxygenase (4.4 μM) was incubated at 20 °C with 50 μM 12-IODE and 50 μM 13-HPOD in 50 mM borate (pH 9.0). After 5 min, glutathione peroxidase and glutathione were added to reduce

[2] We have found that when a solution of 0.6 μM ferrous lipoxygenase was treated with 0.5 equiv of 13-HPOD, the fluorescence change (Egmond et al., 1975) due to the oxidation of 0.3 μM ferrous enzyme to the ferric form was complete as soon as the reactants had been manually mixed. The oxidation reaction is expected to be even faster as the higher concentrations of the experiment described in the text. The stopped-flow fluorescence studies of Aoshima et al. (1977) also imply that oxidation of the ferrous enzyme by 13-HPOD should be very rapid under the conditions of this experiment.

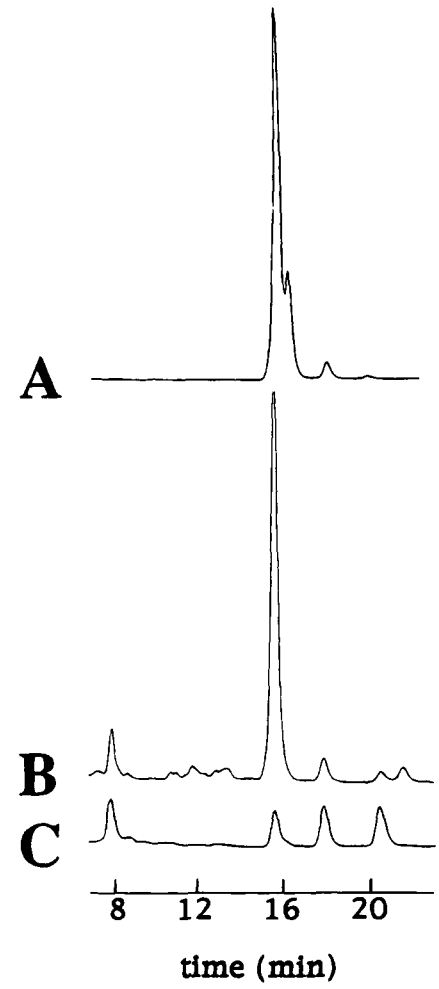


FIGURE 3: HPLC of synthetic 9,11-octadecadienoic acid and the major product from the action of ferric lipoxygenase on 12-IODE. HPLC was carried out using system A with UV detection at 234 nm. (A) Synthetic 9,11-octadecadienoic acid (20-μL injection of a 0.2 mM solution in ethanol). (B) Products from an enzymatic reaction carried out as described under Results and worked up as described under Experimental Procedures. (C) Products from a control experiment identical to that in panel B but lacking enzyme. In each chromatogram, the detector sensitivity was 0.2 AUFS, and the data were plotted with a Spectra Physics SP4290 integrator, which was set at an attenuation of 64. Time zero is the time of injection; the void volume elution and the elution of 13-HOD at 4.7 min in B and C are not shown in the chromatograms.

excess 13-HPOD to 13-HOD, and the reaction mixture was acidified and extracted with ethyl acetate as described under Experimental Procedures. Reverse phase HPLC of the extracted material in system A showed a peak due to 13-HOD at 4.7 min and a peak at 15.6 min (Figure 3B), which had the same mobility as the major component of a synthetic sample of 9,11-ODA (Figure 3A). As described under Experimental Procedures, the synthetic material was prepared by a nonenzymatic elimination reaction and characterized as a mixture of two isomers, of which the major one is 9-*cis*,11-*trans*. The second isomer in the synthetic sample (probably 9-*cis*,11-*cis*) gives rise to a partially resolved peak at 16.3 min in Figure 3A; the absence of this peak from Figure 3B imples that the product of the enzymatic reaction is of higher stereochemical purity than the synthetic sample. Very little 9,11-ODA was detected in a control lacking enzyme (Figure 3C) or in a control lacking 13-HPOD (data not shown). HPLC in system B gave the same result: one

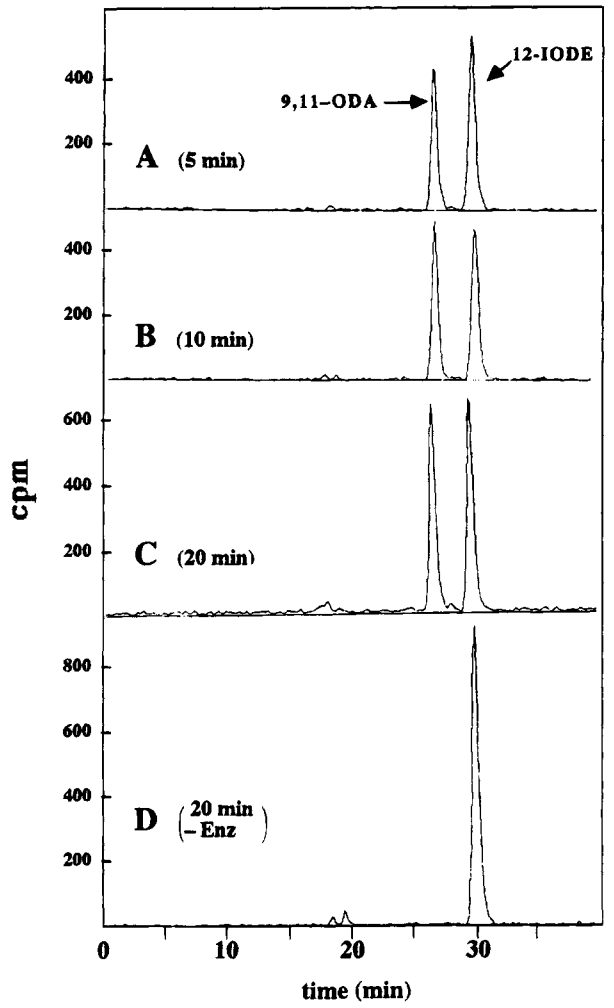


FIGURE 4: Reaction of [1-^{14}C]-12-IODE (66 μM) with lipoxygenase (4.4 μM) in the presence of 13-HPOD (50 μM) monitored by HPLC with radiochemical detection at (A) 5 min, (B) 10 min, and (C) 20 min. (D) Control lacking enzyme at 20 min. Time zero on the abscissa of each chromatogram was the time of injection. HPLC was carried out in system B; each chromatogram was obtained on a 500-μL injection of a 1:1 mixture of reaction mixture and quench solution (see Experimental Procedures). The update time on the radioactive flow detector was 6 s and the background offset was 30 cpm. The integrated intensities of the 9,11-ODA peaks in each chromatogram are (A) 2635 cpm, (B) 2897 cpm, and (C) 4010 cpm. The integrated intensities of the 12-IODE signals are (A) 3558 cpm, (B) 3184 cpm, and (C) 4387 cpm. The average values (±SDEV) of cpm(9,11-ODA)/cpm(12-IODE) for this and a second set of chromatograms are (A) 0.77 ± 0.04, (B) 0.91 ± 0.01, and (C) 0.94 ± 0.04.

major product with the same mobility (30.3 min) as the 9-*cis*,11-*trans* isomer of synthetic 9,11-ODA.

To search for additional products that do not absorb in the UV, [1-^{14}C]-12-IODE (66 μM, 0.44 μCi/μmol) was incubated with lipoxygenase (4.4 μM) and 13-HPOD (50 μM). Aliquots were withdrawn periodically, quenched as described under Experimental Procedures, and analyzed by HPLC using system B with radiochemical detection (Figure 4). After 5 min (Figure 4A), the only significant product was 9,11-ODA, and after 10 min, the peak due to 9,11-ODA was nearly equal in intensity to that due to 12-IODE. After 20 min, the relative intensities of the 12-IODE and 9,11-ODA peaks had not changed appreciably from those at 10 min, even though the enzyme still had about 50% of its catalytic activity. This observation provides strong evidence

Table 1: Formation of 9,11-ODA from 12-BrODE and 12-IODE[a]

substrate	time (min)	9,11-ODA formed (μM) +enz	−enz	net[b]
12-BrODE	4	5.6 ± 1.9	1.3 ± .2	4.3 ± 1.9
12-BrODE	10	9.9 ± 1.6	1.4 ± .2	8.5 ± 1.6
12-IODE	4	20 ± 3	1.0 ± .3	19 ± 2
12-IODE	10	23 ± 2	1.0 ± .3	22 ± 2

[a] Lipoxygenase (4.4 μM) was incubated at 20 °C with either 50 μM 12-BrODE or 50 μM 12-IODE in the presence of 50 μM 13-HPOD in 50 mM borate (pH 9.0). At the indicated times 0.50-mL aliquots were withdrawn, quenched as described under Experimental Procedures, and analyzed by HPLC in system B with UV detection at 234 nm. The concentrations of 9,11-ODA were determined by comparison of the integrated area under the peak with standards of known concentration. The data presented are averages (±SDEV) from two experiments. [b] Difference between the amounts of 9,11-ODA formed in the presence (+enz) and absence (−enz) of lipoxygenase.

that only one enantiomer of racemic 12-IODE is processed by the enzyme. Panels B and C in Figure 4 show very weak signals with elution times of 18−19 min. On the basis of their elution times, the materials giving rise to these signals appear to be considerably more polar than 9,11-ODA. These signals become more prominent at longer incubation times, and they appear to arise partly from nonenzymatic hydrolysis of 12-IODE (see Figure 4D) and partly from a slow, lipoxygenase-catalyzed breakdown of 9,11-ODA. The existence of the latter reaction has been confirmed by incubating lipoxygenase with synthetic 9,11-ODA, and this reaction is currently under investigation.

Similar results were obtained when the lipoxygenase-catalyzed reaction on [1-^{14}C]-12-IODE was monitored using the methodology of the experiment in Figure 3 (extraction with ethyl acetate followed by HPLC with system A). At short reaction times, the only radioactive product was 9,11-ODA; at longer times, more polar radioactive products (retention times = 3−6 min in system A) became detectable.

To determine whether the conversion of 12-IODE to 9,11-ODA requires catalytically active lipoxygenase, 66 μM [1-^{14}C]-12-IODE and 50 μM 13-HPOD were incubated with a sample of lipoxygenase (4.4 μM) that had been inactivated by ETYA (Kuhn et al., 1984) to about 5% of its original specific activity. HPLC analysis after 5 min revealed that only 3% of the [1-^{14}C]-12-IODE had been converted to [1-^{14}C]-9,11-ODA, compared with 37% conversion in a parallel experiment with active enzyme.

pH Dependence. Inactivation of lipoxygenase by 12-IODE is considerably less effective at pH 7.6 than at pH 9. When an experiment identical to that depicted in Figure 1 was carried out at pH 7.6, enzyme that was incubated with 12-IODE for 20 min lost only about 10% of its catalytic activity, relative to a control from which 12-IODE was omitted. In contrast, the production of iodide from 12-IODE occurred at about the same rate at pH 7.6 as at pH 9.0. HPLC experiments indicate that the formation of 9,11-ODA from 12-IODE at pH 7.6 also occurs at about the same rate as at pH 9.0. Formation of iodide from 12-IODE does not occur at pH 6.0.

Experiments with 12-BrODE. When 12-BrODE was incubated with 13-HPOD and lipoxygenase, 9,11-ODA was formed more slowly than from 12-IODE (Table 1). The conversion of 12-BrODE to 9,11-ODA could not be detected if 13-HPOD was omitted or if the enzyme had been pretreated with ETYA. No inactivation by 12-BrODE could

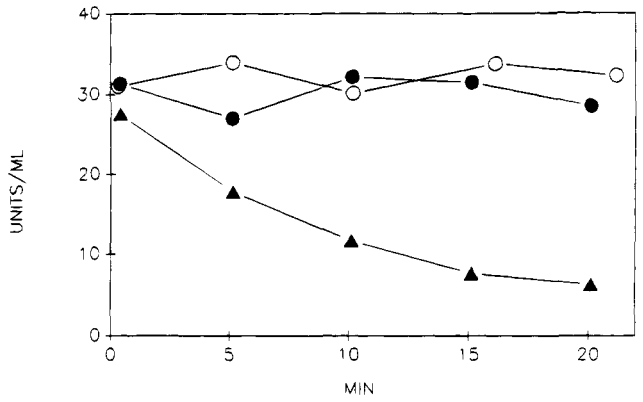


FIGURE 5: Protection of lipoxygenase by ascorbate from inactivation by 12-IODE. Lipoxygenase (1.8 μM) was incubated with 12-IODE (50 μM) and 13-HPOD (51 μM) in the presence of 100 μM ascorbate (●). Controls were carried out lacking ascorbate (▲) and lacking 12-IODE (○). Each experiment was carried out at 20 °C in 50 mM borate (pH 9.0) containing 1% (v:v) ethanol. The experiments were initiated by adding enzyme, and 2.0-μL aliquots were withdrawn at the indicated times and added to 2.5 mL of assay mix to determine lipoxygenase activity.

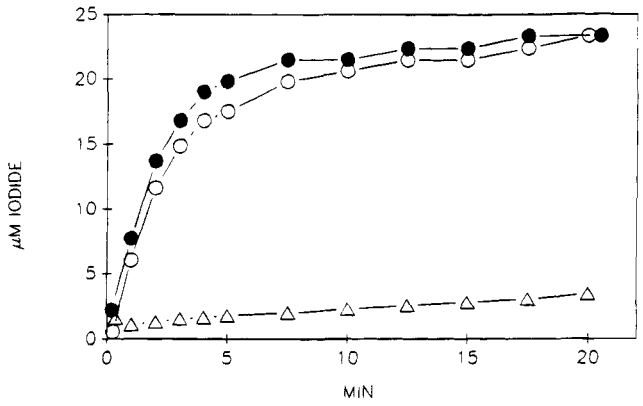


FIGURE 6: Effect of ascorbate on the lipoxygenase-catalyzed release of iodide ions from 12-IODE. Lipoxygenase (4.4 μM) was incubated with 12-IODE (50 μM) and 13-HPOD (51 μM) in the presence (●) and absence (○) of 100 μM ascorbate, and the iodide concentration was determined as described under Experimental Procedures. (△) Control containing 12-IODE (50 μM), 13-HPOD (51 μM), and ascorbate (100 μM).

be detected, even at long incubation times. For example, when lipoxygenase (0.18 μM) was incubated with 50 μM 12-BrODE and 1 μM 13-HPOD at 20 °C, the activity after 1 h was >90% of the initial activity and indistinguishable from the activity of a control lacking 12-BrODE.

Experiments with Sodium Iodide. No loss of activity occurred when 0.18 μM lipoxygenase was incubated for 20 min with 1.0 mM sodium iodide in the presence of 13-HPOD (5 or 50 μM) or in the absence of 13-HPOD.

Effects of Free-Radical Scavengers. The data in Figure 5 demonstrate that 100 μM ascorbate protects lipoxygenase from inactivation by 12-IODE. In contrast, 100 μM ascorbate does not inhibit either the lipoxygenase-catalyzed release of iodide ions from 12-IODE (Figure 6) or the conversion of [1-^{14}C]-12-IODE to [1-^{14}C]-9,11-ODA (data not shown). Since ascorbate is known to be a good free-radical scavenger (Frei et al., 1989), these results suggest that inactivation by 12-IODE involves radicals that are accessible to free-radical trapping agents in solution. Scavengeable radicals do not appear to be involved in the conversion of 12-IODE to 9,11-ODA and iodide.

Since ascorbate is a reducing agent, it could conceivably protect the enzyme by reducing ferric lipoxygenase to the ferrous form, which is not inactivated by 12-IODE. This scenario, however, would be expected to inhibit the conversion of 12-IODE to iodide and 9,11-ODA, since ferrous lipoxygenase does not catalyze this reaction. In addition, ESR studies in our laboratory on the reduction of ferric lipoxygenase by ascorbate demonstrate that this reaction is negligible at 100 μM ascorbate.[3] We therefore conclude that ascorbate is blocking inactivation by intercepting a radical or some other reactive intermediate.

We wished to corroborate our interpretation of the above experiments using other free-radical scavengers. Unfortunately, the most common antioxidants (e.g., BHT) are phenols, which are known to readily reduce ferric lipoxygenase (Kemal et al., 1987). We have previously found that *N*-alkylhydroxylamines rapidly reduce ferric lipoxygenase (Clapp et al., 1985), but hydroxylamine itself does so much less rapidly. Using fluorescence (Kemal, 1987), we have found that 200 μM hydroxylamine reduces 1 μM lipoxygenase in a pseudo-first-order process with $t_{1/2} = 4.5$ min. Since the oxidation of ferrous lipoxygenase by 13-HPOD is very rapid (Aoshima et al., 1977), it should be possible to maintain lipoxygenase in the ferric form in the presence of 200 μM hydroxylamine by using excess 13-HPOD. Although hydroxylamine is not widely used as a free-radical trap, the stability of nitroxide radicals (Roberts, 1979) suggests that it would be effective in this capacity. Hydroxylamine has been used to quench the tyrosyl radical in *Escherichia coli* ribonucleotide reductase (Ehrenberg & Reichard, 1972).

The results in Figure 7A demonstrate that 200 μM hydroxylamine reduces, by about 75%, the inactivation that occurs when 1.8 μM lipoxygenase is incubated with 50 μM 12-IODE in the presence of 50 μM 13-HPOD. Note that there is ample 13-HPOD present (25-fold excess over enzyme) to maintain the enzyme in the ferric form over the time period of this experiment. Figure 7B shows data from the same experiment that demonstrates that hydroxylamine does not inhibit the formation of iodide from 12-IODE. Iodide formation is actually faster in the presence of hydroxylamine, as would be expected if the enzyme is being protected from inactivation. A similar effect may be occurring in the experiment with ascorbate, but it is more difficult to discern in Figure 6 where the enzyme concentration (4.4 μM) is higher than that in Figure 7 (1.8 μM).

Thiols are also capable of trapping free radicals (Ross et al., 1985). The presence of 200 μM dithiothreitol completely blocked the inactivation of 2.6 μM lipoxygenase by 50 μM 12-IODE in the presence of 50 μM 13-HPOD. The effect of dithiothreitol on product formation has not been investigated. It is unlikely that dithiothreitol is protecting the enzyme by reducing it, since experiments in our laboratory, as well as published data (Kemal et al., 1987), indicate that ferric lipoxygenase is reduced less rapidly by dithiothreitol than by hydroxylamine. Interestingly, 250 μM glutathione does not protect lipoxygenase from inactivation by 12-IODE.

Inactivation by [1-^{14}C]-12-IODE. To determine whether the carbon skeleton of 12-IODE becomes covalently attached to inactivated enzyme, 8.8 μM lipoxygenase (specific activity = 188 units/mg) was incubated with 110 μM [1-^{14}C]-12-

[3] M. Xu and C. Clapp, unpublished results.

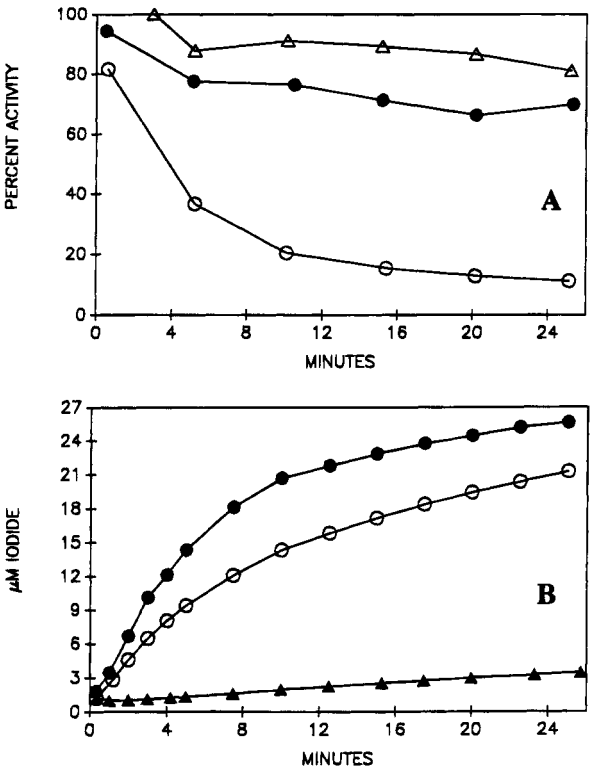


FIGURE 7: Protection of lipoxygenase by hydroxylamine from inactivation by 12-IODE (A) and the effect of hydroxylamine on the lipoxygenase-catalyzed release of iodide ions from 12-IODE (B). Lipoxygenase (1.8 μM) was incubated with 12-IODE (50 μM) and 13-HPOD (50 μM) in the presence (●) and absence (○) of 200 μM hydroxylamine in 50 mM borate (pH 9.0) containing 0.8% (v:v) ethanol. Lipoxygenase activity and iodide concentration were monitored simultaneously, as in Figure 1. As a control in panel A, lipoxygenase (1.8 μM) was incubated with 13-HPOD (50 μM) and hydroxylamine (200 μM) in the absence of 12-IODE (△). As a control in panel B, hydroxylamine (200 μM) was incubated with 12-IODE (50 μM) and 13-HPOD (50 μM) in the absence of lipoxygenase (▲).

IODE (9.8×10^5 dpm/μmol) and 50 μM 13-HPOD in 1.0 mL of 50 mM borate (pH 9.0). The catalytic activity dropped quickly and stabilized at 54% of the initial value after 10 min. The reaction mixture was subjected to gel filtration as described under Experimental Procedures, and the protein fraction was found to contain 8.4×10^3 dpm/mg protein. Most of the radioactivity in the protein fraction could be removed by extraction with ethyl acetate, which reduced the radioactivity level to 670 dpm/mg. This value could be further reduced to 350 dpm/mg by dialysis overnight at room temperature against 7 M guanidine hydrochloride.

Stoichiometric labeling of the enzyme by [1-^{14}C]-12-IODE would correspond to 1.03×10^4 dpm/mg. Considering that 46% of the enzyme in the above experiment was inactivated, incorporation of 1 molecule of inactivator per molecule of enzyme inactivated would lead to 4.7×10^3 dpm/mg, if the enzyme that we started with was fully active.[4] If we assume that our enzyme preparation is only 70% active, by comparison of its specific activity with the highest value (270 units/mL) reported in the literature (Nelson & Cowling, 1990), the corrected value for 1:1 incorporation of inactivator is 3.3×10^3 dpm/mg. In the experiment described in the previous paragraph, the amount of radioactivity that remained in the protein after ethyl acetate extraction was 21% of the corrected value for 1:1 incorporation. The corresponding percentages in two similar experiments were 26% and 28%. From the results of these experiments, we conclude that most of the enzyme molecules inactivated by 12-IODE do not covalently incorporate the carbon skeleton of the inactivator.

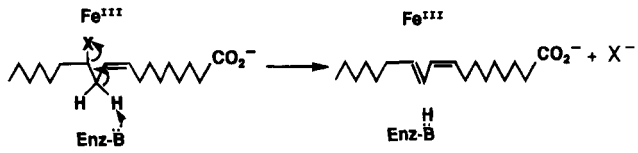
The ethyl acetate extracts from the above experiment were concentrated, mixed with unlabeled 12-IODE and ricinoleic acid, and subjected to thin-layer chromatography over 13 cm on a silica gel plate with hexanes/ethyl acetate/acetic acid (70:30:2) as the mobile phase. Spots due to 12-IODE and ricinoleic acid were visualized by exposure to iodine vapor, and radioactivity was located by scraping 0.5-cm sections of the silica gel from the plate and counting in scintillation fluid. Only 5% of the radioactivity chromatographed with 12-IODE ($R_f = 0.48$), which has the same mobility as 9,11-ODA in this system. Most of the radioactivity ran in an unresolved band between ricinoleic acid ($R_f = 0.31$) and the origin. These results and similar findings with other chromatographic systems indicate that the extracted radioactive material is a complex mixture of substances that are more polar than 12-IODE and 9,11-ODA. This material amounts to only about 5% of the radioactivity initially present in the [1-^{14}C]-12-IODE and could be due to a minor pathway that escaped detection in our product studies.

Although ethyl acetate extraction efficiently removes most of the noncovalently bound radioactivity derived from [1-^{14}C]-12-IODE that elutes with the protein during gel filtration, it does not restore catalytic activity. In one experiment, a single extraction with ethyl acetate removed about 87% of the radioactivity, but reduced the catalytic activity by 17%, which is comparable to the loss of activity that occurs when native enzyme is subjected to ethyl acetate extraction. This result implies that the noncovalently bound material is not responsible for inactivation. Further support for this conclusion comes from experiments at pH 7.6 in which inactivation by 12-IODE is greatly reduced, but noncovalent incorporation of radioactivity is virtually the same as that at pH 9.0. For example, when lipoxygenase (8.8 μM) was incubated with [1-^{14}C]-12-IODE (120 μM) and 13-HPOD (50 μM) in 50 mM Tris (pH 7.6) for 15 min, only 15% of the enzymatic activity was lost, but the protein pool from gel filtration contained 1.1×10^4 dpm/mg, which was reduced to 740 dpm/mg by three extractions with ethyl acetate.

Interestingly, the noncovalent incorporation of radioactivity from [1-^{14}C]-12-IODE into lipoxygenase is greatly reduced by ascorbate. In an experiment at pH 9.0 that was identical to those described in the preceding paragraphs, except that 100 μM ascorbate was present, only 10% of the catalytic activity was lost, and the protein pool after gel filtration contained 680 dpm/mg. This result suggests that the noncovalently bound materials may be formed by a pathway involving radicals, possibly the same pathway that leads to inactivation. However, in light of the evidence presented in the previous paragraph, it is unlikely that the binding of these materials is directly responsible for the loss of activity.

[4] Fluorescence titration of our enzyme preparation with 13-HPOD suggests that 90% of the enzyme molecules are redox-active. Oxidation of ferrous lipoxygenase to the ferric form with 13-HPOD results in a 40% reduction of the fluorescence emission at 327 nm following excitation at 280 nm (Egmond et al., 1975). With the enzyme used in these experiments, 0.9 equiv of 13-HPOD was required to reduce the fluorescence to its minimum value.

Scheme 2



DISCUSSION

In the normal reaction catalyzed by soybean lipoxygenase, the enzyme catalyzes the breaking of a bond between C(11) of linoleic acid and one of the hydrogens attached to that carbon. The enzyme therefore might be expected to catalyze an elimination reaction on substrate analogues in which the C(12)−C(13) double bond of linoleic acid was replaced with a potential leaving group at C(12). The work presented here demonstrates that such a reaction does occur with 12-IODE and 12-BrODE. These elimination reactions are slow, but they appear to be related to the normal activity of the enzyme, since they are not catalyzed by lipoxygenase that has been inactivated by ETYA. It has been shown that the methyl ester of ETYA irreversibly inactivates lipoxygenase without becoming covalently attached to it (Kuhn et al., 1984). By making the reasonable assumption that the same is true for ETYA, one can surmise that the substrate binding site of lipoxygenase that has been inactivated by ETYA is free of inactivator, but is not catalytically active, possibly due to the conversion of a methionine residue to methionine sulfoxide (Kuhn et al., 1984). Our results demonstrate that this defective active site is unable to convert 12-IODE or 12-BrODE to 9,11-ODA.

The first step in the normal reaction catalyzed by ferric lipoxygenase may involve the removal of a proton from C(11) by an enzymic base concomitant with either the transfer of one electron to the iron (Scheme 1a) or the formation of an iron−carbon bond (Scheme 1b) (Corey & Nagata, 1987). The simplest possible mechanism for the elimination reactions reported here is that the same enzymic base catalyzes the cleavage of the C(11)−H bond, concomitant with the breaking of the C(12)−X bond (Scheme 2).

The elimination reactions cannot be detected in the absence of 13-HPOD. This result implies that ferrous lipoxygenase cannot catalyze these reactions. On the basis of the mechanistic suggestion in the previous paragraph, it might be expected that the elimination would occur with either the ferrous or ferric form of the enzyme, since the iron is not involved in the reaction. The failure of the ferrous enzyme to catalyze elimination might result from a conformational difference between the ferrous and ferric enzymes. Alternatively, it is possible that the mechanism of the elimination reaction is more complex than that shown in Scheme 2 and utilizes the redox properties of the iron. At present, it is not possible to distinguish between these alternatives.

There is general agreement that ferric lipoxygenase is catalytically active toward normal substrates, but the question of whether the ferrous form is also active has been controversial (Wang et al., 1993; Schilstra et al., 1994). Although the work reported here does not bear directly on this question, the failure of the ferrous enzyme to catalyze elimination reactions demonstrates a significant difference in the catalytic capabilities of the two forms.

On the basis of the yields after 4 min in Table 1, the lipoxygenase-catalyzed conversion of 12-IODE to 9,11-ODA appears to be about 4 times faster than the corresponding reaction with 12-BrODE. The actual rate difference is somewhat greater than this, since the reaction with 12-IODE is nearing completion after 4 min, and since the enzyme undergoes significant inactivation in the presence of 12-IODE, but not in the presence of 12-BrODE. The higher rate with 12-IODE probably reflects the superiority of iodide to bromide as a leaving group. By comparison, the E2 elimination of HI from phenethyl iodide at 30 °C is 6.5 times faster than the corresponding reaction with phenethyl bromide (DePuy & Froemsdorf, 1957). Since iodine is larger than bromine, it is possible that 12-IODE has greater difficulty than 12-BrODE binding to the active site of lipoxygenase. The fact that lipoxygenase-catalyzed elimination is faster for 12-IODE than for 12-BrODE, in spite of the greater size of the iodine atom, implies that breaking the carbon−halogen bond must be at least partially rate-limiting in these reactions.

As previously reported, 12-IODE, but not 12-BrODE, irreversibly inactivates soybean lipoxygenase in the presence of 13-HPOD. Neither 9,11-ODA (Rotenberg et al., 1988) nor iodide inactivates the enzyme. The inactivation by 12-IODE can be blocked by ascorbate, hydroxylamine or dithiothreitol. All three of these compounds are capable of acting as free radical traps, and their ability to block inactivation suggests that a pathway exists by which lipoxygenase can convert 12-IODE to a radical, which then attacks the enzyme. Alternatively, since all three of the aforementioned compounds are reducing agents, it is possible that inactivation involves a reactive nonradical species, such as an iodoso compound, that can be quenched by reduction.

When the enzyme is inactivated by [1-^{14}C]-12-IODE, the amount of radioactivity that becomes covalently attached to the protein is less than 30% of the value that would be expected for stoichiometric labeling. This finding, in light of the results with ascorbate, hydroxylamine, and dithiothreitol, suggests that the major pathway leading to inactivation involves an attack on the enzyme by a reactive intermediate that can irreversibly modify lipoxygenase without becoming covalently attached to the enzyme. A radical could accomplish this by the removal of a hydrogen atom or an electron from a group at the active site. This process would create a radical site on the enzyme, which would likely react with O_2 to lead to irreversible modification. Free-radical traps could block inactivation either by quenching the 12-IODE-derived radical before it attacks the enzyme or by transferring an electron or a hydrogen atom to the radical site on the enzyme before it reacts with O_2.

In sum, the results presented here imply that lipoxygenase processes 12-IODE by two pathways. The first is elimination to produce iodide and 9,11-ODA; the second leads to inactivation and proceeds via an intermediate such as a radical that can be trapped by ascorbate or hydroxylamine. The second pathway apparently is unavailable to 12-BrODE. This observation suggests that the second pathway might involve oxidation on the iodine atom or homolysis of the relatively weak C−I bond. Studies on the second pathway and its mechanistic relationship to the first are continuing in our laboratory.

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