Role of Lipid Hydroperoxides in the Activation of 15-Lipoxygenase†

Gareth D. Jones,‡ Linda Russell,§ Victor M. Darley-Usmar,|| David Stone,§ and Michael T. Wilson*,‡

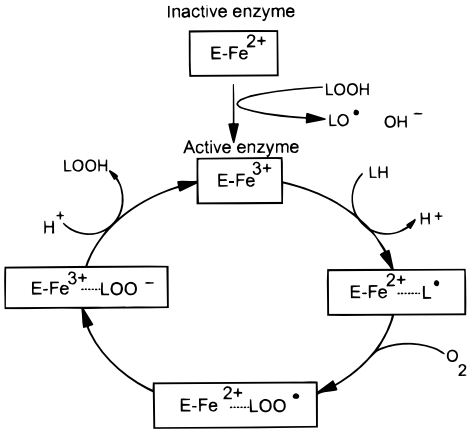
Department of Biological and Chemical Sciences, University of Essex, Colchester, Essex CO4 3SQ, United Kingdom, Biology Division, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, United Kingdom, and Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019

Received October 9, 1995; Revised Manuscript Received February 7, 1996⊗

ABSTRACT: We have used stopped-flow rapid reaction methods, employing both fluorescence and absorbance monitoring, together with HPLC analysis of the products to study the activation of soybean 15-lipoxygenase by 13(*S*)-hydroperoxy-9,11(*E*,*Z*)-octadecadienoic acid (13-HPOD). When lipoxygenase is mixed with an equimolar concentration of 13-HPOD, the enzyme undergoes a rapid change in fluorescence. The rate of the change of fluorescence is dependent on the concentration of the 13-HPOD ($k = 6.7 \times 10^6$ M^{-1} s^{-1}) and is accompanied by activation of the enzyme. The fluorescence change is not accompanied by any change in the UV absorbance of the 13-HPOD, suggesting no loss of the conjugated diene during enzyme activation, and HPLC analysis of the products of the reaction confirms that the 13-HPOD can be recovered unchanged following this reaction. In the presence of an inhibitor (BWA4C, a hydroxamate inhibitor) that reduces the active-site iron, the 13-HPOD and the inhibitor are destroyed in a peroxidase-like reaction. On the basis of these observations we propose that 13-HPOD binds to the enzyme and facilitates activation of the enzyme, possibly through the formation of a protein radical, and that the 13-HPOD is not changed chemically in this process.

The lipoxygenase enzymes are widespread in biological systems and the products they form play a key role in modulating the inflammatory process and remodeling membranes (Veldink et al., 1977; Rapoport et al., 1979; Kuhn et al., 1990; Ford-Hutchinson, 1991). They oxygenate lipids stereospecifically to form stable peroxide groups which are then transformed by other enzymes in the cell to form an impressive array of lipid oxidation products with a wide range of biological properties (Schewe et al., 1986; Salmon, 1986). A family of lipoxygenases have been identified which show varying degrees of chemical specificity with respect to the lipid substrates they may oxidize and, therefore, the products they form (Schewe et al., 1986; Yamamoto, 1992). Despite differences in the lipid substrates oxidized and control of the posttranscriptional activation process, the catalytic mechanism of action appears to be conserved throughout this range of diverse proteins. The enzyme which has been readily available for mechanistic studies for some 30 years is the soybean 15-lipoxygenase, so called because it is capable of oxygenating arachidonic acid to form 15-HPETE[1] (Kuhn et al., 1986). The enzymes contain a single non-heme iron which mediates the necessary electron transfer reactions to achieve lipid peroxide formation without the release of lipid-derived free radicals into the cell (De Groot et al., 1975).

Scheme 1: Mechanism for the Oxygenation of Lipids by Lipoxygenase under Aerobic Conditions



The essential features of a current hypothesis for the mechanism of catalysis are shown in Scheme 1 (De Groot et al., 1975). When prepared the enzyme is inactive and the iron is in a form which is ESR-silent and has a Mossbauer spectrum characteristic of a ferrous oxidation state (E-Fe^{2+}) (De Groot et al., 1975; Pistorius & Axelrod, 1976). Numerous kinetic studies have shown that, for the enzyme to exhibit activity, pretreatment with a stoichiometric quantity of a lipid hydroperoxide (LOOH) is required (Smith & Lands, 1972; Egmond et al., 1977; Ludwig et al., 1987; Schilstra et al., 1992, 1994). Once the enzyme is in the ferric oxidation state (E-Fe^{3+}) it catalyzes a stereospecific abstraction of a H atom from a bis-allylic carbon atom in the fatty acid side chain to form the potentially highly reactive alkyl radical (L·), which remains bound to the protein. In this step the iron in the enzyme is reduced from the ferric to the ferrous oxidation state. Oxygen then reacts with the alkyl radical to form the peroxyl radical (LOO·), which is subsequently reduced by the iron to form the peroxyl anion (LOO^-), which, after protonation, is released as a lipid hydroperoxide (LOOH).

† This work was supported in part by NIH Grant HL 48676-4 to V.M.D.-U. M.T.W. acknowledges funding by BBSRC (U.K.).

* Author to whom correspondence should be addressed.
‡ University of Essex.
§ Wellcome Research Laboratories.
|| University of Alabama at Birmingham.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-9,11(*E*,*Z*)-octadecadienoic acid; 9-HOD, 9(*S*)-hydroxy-10,12(*E*,*Z*)-octadecadienoic acid; 13-HOD, 13(*S*)-hydroxy-10,12(*E*,*Z*)-octadecadienoic acid; 15-HPETE, 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; $15L_s$-HETE, 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; HPLC, high-performance liquid chromatogaphy; ESR, electron spin resonance; BWA4C, *N*-[(*E*)-3-(3-phenoxyphenyl)prop-2-enyl]acetohydroxamic acid.

S0006-2960(95)02425-1 CCC: $12.00

The precise mechanism of the activation process has not been fully elucidated. Interaction of the hydroperoxide with the enzyme results in the production of an ESR signal which has some of the characteristics of ferric iron and this has led to the suggestion that the peroxide oxidizes the enzyme to the ferric form (DeGroot et al., 1975). These changes are also associated with the quenching of the intrinsic fluorescence of the protein (Finazzi-Agro et al., 1973; Egmond et al., 1975), and this process has been used as a means to monitor activation of the enzyme prior to turnover (Wang et al., 1993; Schilstra et al., 1994). The product of this activation reaction would necessarily be lipid alkoxyl radical (LO•) derived from the priming lipid hydroperoxide. A range of products consistent with such a scheme are formed when the enzyme is activated under anaerobic conditions in the presence of linoleic acid (De Groot, 1975). If the activation of the enzyme by the product occurs as part of the catalytic cycle of lipoxygenases, then the enzymes would act as a peroxidase and in fact certain classes of inhibitors, which appear to reduce the iron in the enzyme directly, do promote the decomposition of lipid peroxides (Reynolds, 1988; Nelson et al., 1991; Falgueyret et al., 1993). Other studies have shown that the consumption of lipid peroxide occurs during the catalytic cycle of the enzyme (Kuhn et al., 1986). However, experiments in which the activation of the enzyme by 13-HPOD in the absence of substrate, monitored under conditions where the fate of the peroxide is followed, have not been undertaken, leaving the role of the hydroperoxidase activity in the normal activation and turnover of the enzyme unclear.

In the present study, we have reexamined the role of lipid hydroperoxides in the activation of soybean 15-lipoxygenase using rapid reaction techniques whereby we can follow the fluorescence or absorbance changes that occur when the enzyme interacts with lipid peroxide, linoleic acid, or a potent inhibitor capable of promoting the peroxidase reaction. The results of these studies, taken together with product analysis by HPLC, suggest a modification of previous schemes for the activation of the enzyme. The essential feature of this scheme is that activation of the enzyme by lipid hydroperoxide does not result in a chemical change in the hydroperoxide which remains bound to the enzyme. The consequences of this conclusion for other aspects of the mechanism are explored.

MATERIALS AND METHODS

Purification of Lipoxygenase. Lipoxygenase 1 was purified from lyophilized soybean lipoxidase type 1 (Sigma) by an adaptation of the method described by Finazzi-Agro et al. (1973). Soybean lipoxidase (150 mg) was suspended in 15 mL of 0.05 M sodium acetate buffer, pH 5.5, containing 0.5 M sodium chloride and then dialyzed against the same buffer. The resulting solution was applied to a CM Sephadex C-50 column (20 cm × 1.5 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.5, containing 0.05 M sodium chloride at a flow rate of 6 mL/h. The enzyme was eluted with a linear gradient of 0.05−0.3 M NaCl in 0.05 M sodium acetate buffer, pH 5.5 (total volume 100 mL). Lipoxygenase activity was measured in the eluant (Kemal *et al.*, 1987), and active fractions were combined and showed a single band on polyacrylamide gel electrophoresis (Phastgel, Pharmacia). The concentration of soybean lipoxygenase was determined by measuring the A_{280nm} of the solution assuming and calculated assuming an extinction coefficient of 1.4×10^5 M^{-1} cm^{-1} at this wavelength (Axelrod et al., 1981). The capacity of preparations to bind 13-HPOD was routinely checked by titrating the enzyme with 13-HPOD and monitoring fluorometrically (Egmond et al., 1975). Preparations typically bound 0.75−1.0 mol of 13-HPOD/mol of enzyme.

Preparation of 13(S)-Hydroperoxyoctadecadien-1-oic Acid. The hydroperoxide was prepared as described by Egmond et al. (1976) with the exception of the procedure for extraction and separation of the final product. Following incubation of linoleic acid with the enzyme, the reaction mixture at pH 3.0 was extracted twice with an equal volume of diethyl ether and the combined ether extracts were washed four times with the same volume of distilled water. The washed ether extract was dried by adding anhydrous magnesium of sulfate (v/w 10/1), which was removed by filtration, and the ether extract was dried down under a stream of nitrogen. The 13-HPOD was separated on a 10-cm × 1-cm column of Mallinckrodt CC4 silica gel (activated at 120 °C for 2 h) equilibrated and eluted with 10% diethyl ether/hexane (v/v). The dried extract was dissolved in 10% diethyl ether/hexane and loaded onto the column, which was then washed with 50 mL of the same solvent to remove any unreacted linoleic acid. The 13-HPOD was then eluted with 50 mL of 20% diethyl ether/hexane (v/v) and its purity was checked by ultraviolet spectroscopy, thin-layer chromatography, and HPLC. The concentration of the 13-HPOD was determined using an ϵ of 25 000 M^{-1} cm^{-1} at 234 nm. The ϵ at 250 nm was calculated to be 10^4 M^{-1} cm^{-1}.

Preparation of [1-^{14C}]-13(S)-Hydroperoxyoctadecadien-1-oic Acid. The method was a scaled-down version of that described above except the linoleic acid starting material comprised 2.5 mg of [1-^{14}C]linoleic acid (50 μCi).

Rapid Kinetic Stopped-Flow Studies. Sodium borate buffer (0.1 M) containing 1% ethanol and 0.1% sodium cholate (Sigma Chemical Co.), pH 9.6, was used in all rapid kinetic stopped-flow experiments unless stated otherwise. The linoleic acid solutions were prepared shortly before use at a stock concentration of 200 μM and contained approximately 3% (6 μM) conjugated diene (assuming an extinction coefficient at 234 nm of 25 000 M^{-1} cm^{-1}). On the basis that the contaminating conjugated diene arises from chemical oxidation of the linoleic acid, we assume that 25% of this contaminant is 13-HPOD. The latter thus represents some 0.8% of the initial linoleic acid concentration at the start of each time course. The Fe^{3+} form of lipoxygenase (yellow enzyme) was prepared immediately before use by adding a 25% molar excess of 13-HPOD to the native enzyme.

Rapid kinetic stopped-flow studies were carried out on a DX-17 MV Applied Photophysics spectrofluorimeter apparatus equipped with a 150-W xenon arc lamp light source. The dead time of the apparatus was estimated to be 1.0 ms. Changes in conjugated diene concentrations were monitored at 250 nm, while changes in fluorescence above 320 nm were monitored using a WG320 cutoff filter in front of the photomultiplier (excitation at 280 nm). In experiments where both fluorescence and absorbance measurements were made, the changeover from fluorescence to absorbance mode was made rapidly (via a software switch) and the photomultipliers were allowed 30 s to stabilize. Thus the fluorescence and absorbance time courses were obtained under identical conditions with consecutive "shots" on the same apparatus. In fluorescence time courses taking ~100 s to complete, slight

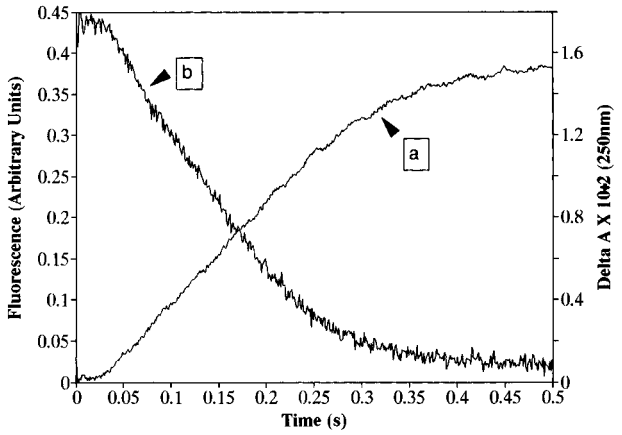


FIGURE 1: Time courses of rapid fluorescence and absorbance changes when native soybean lipoxygenase reacts with linoleic acid. Linoleic acid (10 μM) and native soybean lipoxygenase (2 μM) were initially present after mixing in the stopped-flow spectrophotometer. The temperature was 24.3 °C. Trace a: time course monitoring absorbance changes at 250 nm (0.2-cm path length, three consecutive experiments averaged); right-hand ordinate applies. Trace b: time course monitoring fluorescence changes (excitation at 280 nm, emission from a 320-nm cutoff filter, two consecutive experiments averaged); left-hand ordinate applies.

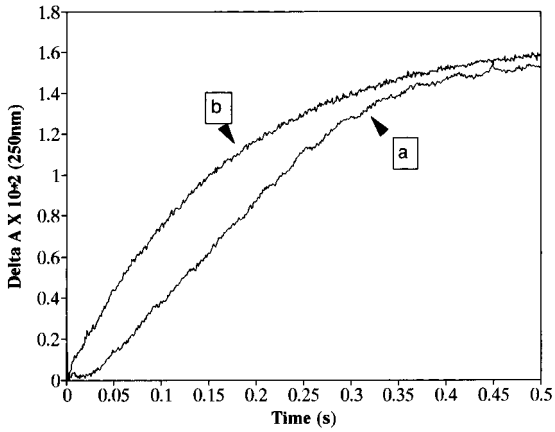


FIGURE 2: Comparison of absorbance changes observed when native and 13-HPOD-treated soybean lipoxygenase react with linoleic acid. Absorbance changes were monitored at 250 nm. Trace a: linoleic acid (10 μM) and soybean lipoxygenase (2 μM) were initially present after mixing (see trace a, Figure 1). Trace b: linoleic acid (10 μM) was reacted with soybean lipoxygenase (2 μM) that had been preincubated with a 25% molar excess of 13-HPOD. Conditions as for Figure 1.

photobleaching was corrected for by using data from control experiments in which the enzyme was mixed with buffer.

Anaerobic conditions in the stopped-flow spectrofluorimeter were achieved by performing several cycles of vigorous degassing followed by N_2 equilibration on the relevant solutions and by carefully transferring these to the drive syringes (O_2 concentrations ≤ 5 μM). To ensure complete anaerobicity in the tubing leading to the mixer and observation chamber, 40 mM sodium dithionite was included in the enclosed temperature control fluid.

HPLC Analyses of Products. Enzyme (2 μM) was incubated for 5 s with 13-HPOD (2 μM) and, as appropriate, linoleic acid (2 μM) in 0.1 M sodium tetraborate, pH 9.3 (incubation volume 2 mL). 9-HOD was included as an internal standard. In contrast to the buffer used for the stopped-flow experiments this buffer did not contain 0.1% cholate since the latter interfered with the subsequent extraction and HPLC analysis. At the end of the incubation, acetic acid (final concentration 0.66 M) was added to reduce the pH to ~4.0 and the samples were extracted with an equal volume of chloroform/ether (1/1). The chloroform layer was dried down under N_2 and dissolved in HPLC running solvent (see below). HPLC was carried out using a Beckman System Gold HPLC system equipped with a 168 diode-array UV detector and a 171 radiochemical detector. Chromatography was carried out on a LiChrospher CN column (Merck) in hexane/propan-2-ol/acetic acid (100/1.25/0.1) at a flow rate of 1.3 mL/min. In some cases concentrations of 13-HPOD were corrected for those of the internal standard 9-HOD assuming complete recovery of the latter.

RESULTS

Rapid Reactions of Lipoxygenase with Linoleic Acid. We have investigated the changes in the fluorescence (emission > 320 nm) and absorbance at 250 nm of a sample of native soybean lipoxygenase that had been mixed rapidly with a 5-fold excess of linoleic acid (Figure 1). A key feature of the time courses is the distinct lag phase that is contemporaneous for both the fluorescence and absorbance time courses. At higher linoleic acid concentrations (25−100 μM) the length of the lag phase remained at about 30 ms and the magnitude and half-times of the fluorescence changes were unchanged and independent of the linoleic acid concentration. The absorbance change at 250 nm, which monitors product formation, increased, as expected, in proportion to the substrate concentration, as did the time taken to complete the conversion of substrate to product. At high substrate concentrations (about 10 μM and thus above the K_m for linoleic acid) the enzyme is saturated with substrate and once activated ($t_{1/2}$ 50 ms) product formation continued until the linoleic acid is depeleted (~3 s at 100 μM substrate). If native lipoxygenase is pretreated with a slight excess of 13-HPOD to produce the form termed by previous workers the "yellow" enzyme, no change in fluorescence is observed when the sample is rapidly mixed with a 5-fold excess of linoleic acid (result not shown). In this case no lag is observed in the appearance of conjugated diene as shown in Figure 2. This figure clearly shows the significant difference in the time courses for conjugated diene production obtained with native or 13-HPOD-treated lipoxygenase under otherwise identical conditions.

Rapid Reactions of Native Lipoxygenase with 13-HPOD: Activation of Native Lipoxygenase. Figure 3 shows typical time courses of fluorescence and absorbance changes at 250 nm seen when 13-HPOD is rapidly mixed with native soybean lipoxygenase. These fluorescence traces show a very rapid process, in the millisecond time range, the rate of which exhibits a dependence on 13-HPOD concentrations. This dependence is depicted in Figure 3B where the apparent rate constant, k_{obs}, is plotted as a function of hydroperoxide concentration. These data conform to a mechanism in which 13-HPOD binds to the enzyme in a second-order process ($k \sim 6.7 \times 10^6$ M^{-1} s^{-1}) which is not saturable over the range of conditions explored in the present study. The time courses also show that when the native enzyme is mixed with an equimolar amount of 13-HPOD, there is no significant loss in the content of conjugated diene as judged by observing the change in absorbance at 250 nm (Figure 3A, trace c). Given that the extinction coefficient of 13-HPOD is 10^4 M^{-1}

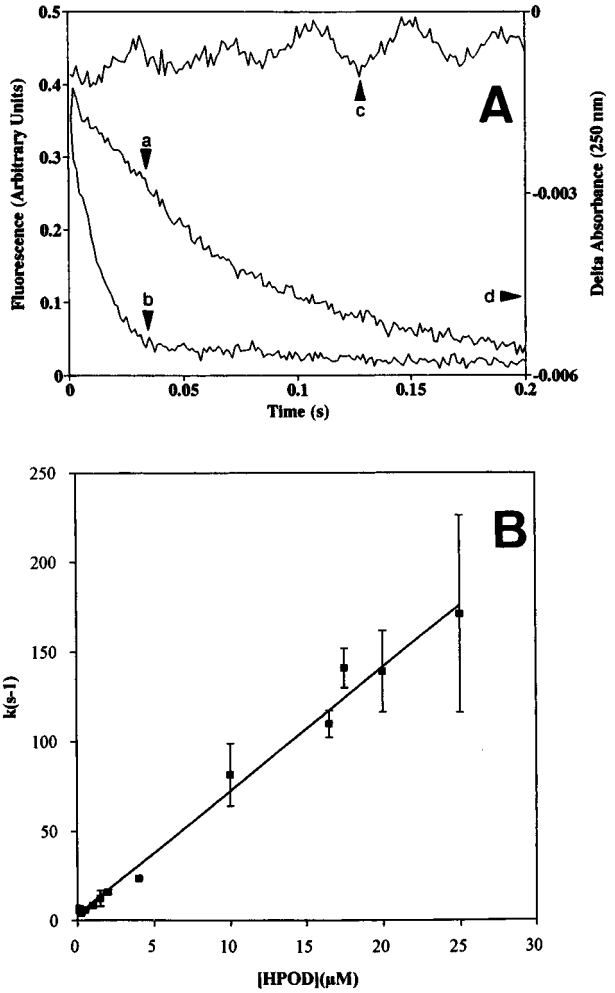


FIGURE 3: (A) Time courses of fluorescence and absorbance changes when lipoxygenase is reacted with 13-HPOD. Rapid fluorescence changes were monitored in a stopped-flow spectrophotometer when approximately 2 μM lipoxygenase (after mixing) reacted with 1.8 μM (trace a) and 10 μM (trace b) 13-HPOD; left-hand ordinate applies. The absorbance at 250 nm was monitored when approximately 2 μMlipoxygenase was reacted with 10 μM 13-HPOD (trace c); right-hand ordinate applies. The temperature was 25 °C. Point d indicates the expected decrease in absorbance if the 13-HPOD was decomposed to a species that did not absorb at 250 nm. (B) 13-HPOD concentration dependence of the rapid fluorescence changes observed when lipoxygenase is rapidly mixed with 13-HPOD. The temperature was 25 °C and the enzyme concentration was approximately 2 μM after mixing. The fit was obtained over an [HPOD] range for which pseudo-first-order kinetics are approximated, i.e., [HPOD] > 5[Enz].

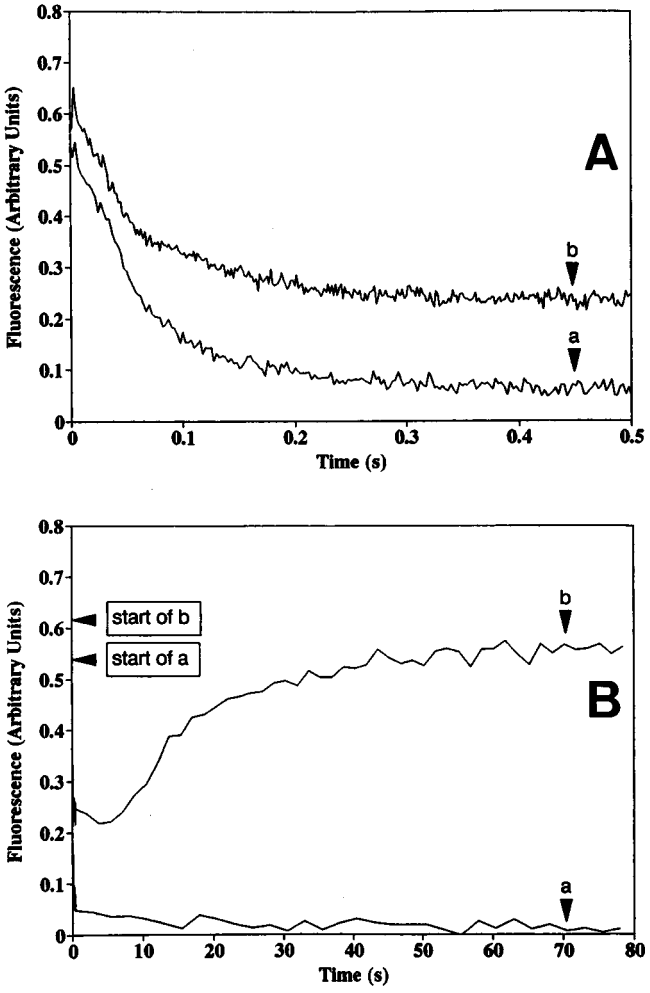


FIGURE 4: Effect of preincubating lipoxygenase with the inhibitor BWA4C on the fluorescence changes observed on mixing with 13-HPOD. Enzyme and reagents were all at a concentration of approximately 2 μM after mixing in the stopped-flow apparatus. (A) Rapid quenching of fluorescence observed when 13-HPOD was mixed with lipoxygenase (trace a) and lipoxygenase preincubated with BWA4C (trace b). A decrease in fluorescence of approximately 0.5 corresponds to a change of approximately 25% of the enzyme's initial fluorescence intensity. (B) Longer time courses monitoring the fluorescence changes of the mixtures described above.

cm^{-1} at 250 nm under the conditions used (see Materials and Methods), there was no apparent loss of 13-HPOD on production of the active enzyme (see Figure 3A, point d). This is an important result and was reexamined using higher enzyme concentrations (8 μM). The results confirmed the conclusion that while mixing the enzyme with 13-HPOD results in rapid fluorescence changes, this was not accompanied by changes in absorbance, i.e., no conjugated diene is lost. When the experiments described above were carried out under strictly anaerobic conditions, identical time courses for the change of fluorescence were observed (data not shown).

Incubation with 13-HPOD plus an Inhibitor. Figure 4 shows a comparison of the reaction of 13-HPOD with native lipoxygenase and with enzyme incubated with an inhibitor, BWA4C. The fluorescence time courses indicate that the rate of fluorescence quenching (and thus activation) of the enzyme remains rapid in the presence of an inhibitor but that the extent of the fluorescence change is significantly diminished. In the presence of the inhibitor this rapid partial activation process is followed by a return of the fluorescence intensity over some 20 s.

HPLC Analysis of the Products of the Reactions of Lipoxygenase with 13-HPOD and with Inhibitor. The activation of the enzyme with 13-HPOD did not result in a loss of absorbance at 250 nm, implying either that HPOD remains unchanged, as there is no loss in conjugated diene, or that other species form which have a similar absorbance at 250 nm. We have therefore examined the products of this reaction by HPLC. The enzyme (2 μM) was incubated with a stoichiometric amount of 13-HPOD under conditions which were very similar to those used for the stopped-flow studies and under which we observed the expected reduction in fluorescence intensity. As shown in Figure 5, the product extracted from these incubations is indistinguishable with regard to its behavior on HPLC from authentic 13-HPOD. The product also has an identical absorption spectrum to the

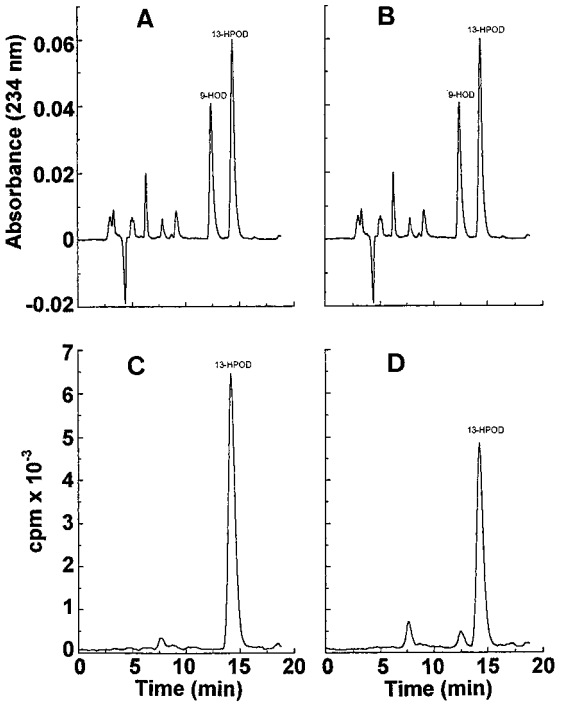


FIGURE 5: HPLC analysis of products of reaction between lipoxygenase (2.0 μM) and 13-HPOD (2 μM). The products were extracted and analyzed by HPLC as described under Materials and Methods. (A) Control incubation in the absence of lipoxygenase. (B) Incubation with lipoxygenase. (C) Control incubation with [1-^{14}C]-13-HPOD and no added lipoxygenase. (D) Incubations of 2.0 μM lipoxygenase and 2 μM [1-^{14}C]-13-HPOD (5 μCi/μmol).

starting material (data not shown). HPLC analyses of incubations carried out using [1-^{14}C]-13-HPOD revealed the presence of some radiolabeled products distinct from the 13-HPOD but these always represented only a minor proportion (0.1−0.2 μM) of the original radioactivity (Figure 5). In a parallel series of experiments we found that this substoichiometric conversion of 13-HPOD (10% or less of the enzyme concentration) was associated with complete fluorescence quenching (result not shown).

In a series of experiments in which 2 μM enzyme was mixed with 2 μM 13-HPOD, the recovery of 13-HPOD, based on the recovery of the UV-absorbing material relative to that of an internal standard (9-HOD), was consistently of the order of 70%, suggesting either that partial degradation of the 13-HPOD occurs or that the recovery of the internal standard did not reflect that of the 13-HPOD. When the same experiment was carried out using a fixed concentration of 13-HPOD (5 μM) and increasing amounts of enzyme, it was clear that the recovery of the 13-HPOD decreased with increasing protein concentration (Figure 6). However, the loss of 13-HPOD was considerably less than equimolar with respect to the amount of active enzyme added (Figure 6, dotted line), suggesting that the failure to recover all of the 13-HPOD reflects an extraction artifact.

In contrast to the results observed with incubations of enzyme and 13-HPOD alone, the addition of inhibitor results in the rapid loss of both 13-HPOD and the inhibitor itself and the appearance of a number of products which can be separated by HPLC (Figure 7). This result confirms that our preparation of lipoxygenase exhibits peroxidase activity in the presence of reducing inhibitors as described by others (Reynolds, 1988; Nelson et al., 1991; Falgueyret et al., 1993).

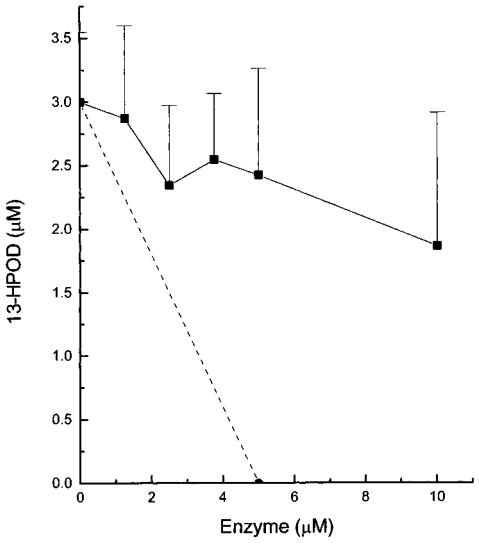


FIGURE 6: Recovery of 13-HPOD from incubations with lipoxygenase. 13-HPOD (5 μM) was mixed with increasing amounts of lipoxygenase and extracted under the conditions described under Materials and Methods. The extracts were analyzed by HPLC (see Figure 5). Results are expressed as the mean ± SEM for three independent experiments. The dotted line represents the expected recovery of 13-HPOD if it were destroyed by interacting with the enzyme on a mole for mole basis.

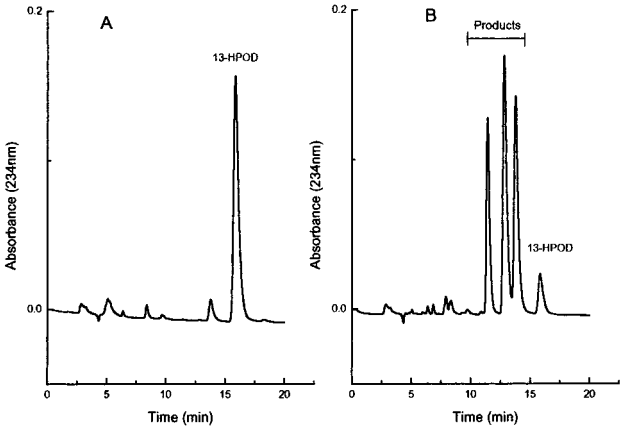


FIGURE 7: HPLC analysis of products obtained by incubation of lipoxygenase (0.1 μM) with 40 μM 13-HPOD, (A) in the absence and (B) in the presence of 40 μM BWA4C, for 10 min in 0.1 M sodium tetraborate (pH 9.3) at 20 °C. Chromatography was carried out as described under Materials and Methods.

DISCUSSION

It has been established that the iron in the native lipoxygenase enzyme is in the ferrous oxidation state and that activation by 13-HPOD is associated with oxidation to a catalytically competent ferric form (De Groot et al., 1975). The ESR data suggest that the interaction of 13-HPOD with the enzyme results in the formation of this ferric form regardless of whether oxygen is present or not, suggesting that the oxidant for this process is either the 13-HPOD or the protein itself (De Groot et al., 1975). Although a pseudoperoxidase reaction has been described for lipoxygenase in the presence of compounds which are capable of reducing the iron from the ferric state, there is limited experimental evidence for such a reaction occurring in the presence of peroxide alone (Reynolds, 1988; Mansuy et al., 1988; Nelson et al., 1991; Falgueyret et al., 1993).

In the present study we have used stopped-flow methods in conjunction with HPLC analysis of products to investigate

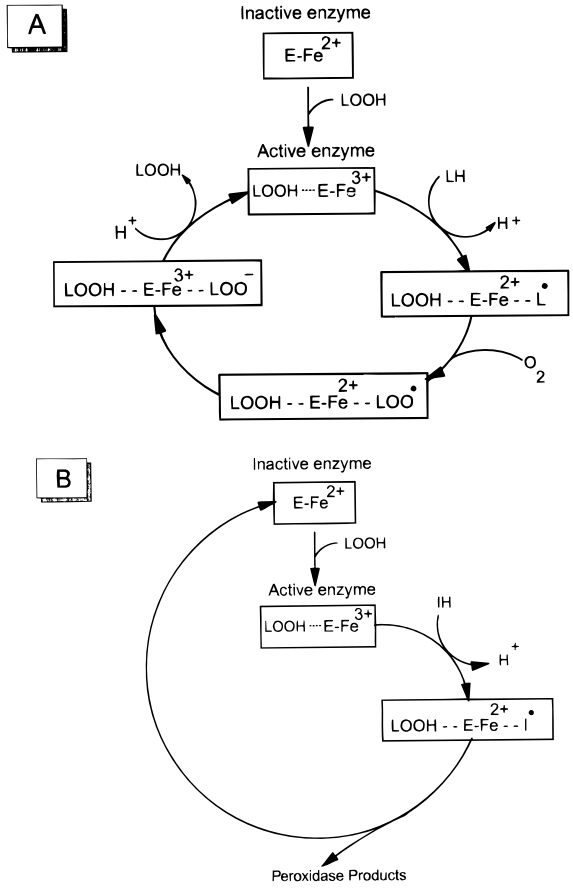
the interaction of both linoleic acid and 13-HPOD with the enzyme. The interaction of 13-HPOD with the native enzyme results in a 30% reduction of the intrinsic fluorescence of the enzyme and is associated with an apparent oxidation of the active-site iron (Egmond et al., 1975; De Groot et al., 1975). Schilstra et al. (1994) have used this property to study the relationship between the activity of the enzyme and its oxidation state and have shown that the activity of the enzyme following activation with varying amounts of 13-HPOD is dependent on the extent of the fluorescence quench and hence by inference on the proportion of the enzyme in the Fe^{3+} state.

In our studies of the interaction of lipoxygenase with linoleic acid we have monitored both the intrinsic fluorescence of the enzyme and the absorbance of the product. Our results (Figures 1 and 2) show that production of 13-HPOD from the inactive (Fe^{2+}) enzyme is preceded by a brief lag phase and that a similar lag is associated with the fluorescence quench. Pretreatment of the enzyme with 13-HPOD eliminates the fluorescence change and abolishes the lag phase for conjugated diene formation. These results are consistent with the hypothesis that the lag phase is associated with the conversion of the Fe^{2+} to Fe^{3+} forms as 13-HPOD accumulates. Our results are in agreement with those reported by Schilstra *et al*. (1994) but contrast with those of Wang *et al*. (1993), who observed a lag in the fluorescence change but no lag in product formation.

We have adopted the same approach of stopped-flow methods using both fluorescence and absorbance measurements to examine more closely the activation of the enzyme by 13-HPOD. The reaction results in a rapid quench of the intrinsic fluorescence of the enzyme, the rate of the process being dependent on 13-HPOD concentration. Over the range of concentrations of 13-HPOD that we have examined (0−25 μM), this process was not saturable. If, as is generally accepted, the fluorescent change in the enzyme is a result of a change in the oxidation state of the active-site iron, it is reasonable to expect that the 13-HPOD (or some other oxidant) should be reduced as a consequence. If the 13-HPOD were the oxidant, then the initial product of the one-electron oxidation would be the corresponding alkoxy radical, which would rapidly rearrange, resulting in the destruction of the conjugated diene and the associated absorbance at 234 nm (Wilcox & Marnett, 1993). For practical reasons we were obliged to follow the absorbance change at 250 nm and at this wavelength no decrease in absorbance was observed. This could have arisen as a consequence of conversion of 13-HPOD to products with similar absorbance characteristics. To test for the presence of such products, 13-HPOD was extracted after incubation with enzyme and quantified by HPLC analysis. According to Scheme 1, where 13-HPOD acts as a one-electron oxidant, one mole of lipid hydroperoxide must be metabolized for each mole of enzyme converted to the ferric form.

In the experiments described here, incubation of a fixed concentration of enzyme with a fixed concentration of 13-HPOD or titration of 13-HPOD with the enzyme did not result in stoichiometric conversion of the hydroperoxide to other products. Such loss of lipid hydroperoxide that did occur could be ascribed to an isolation artifact consequent from the presence of a high concentration of protein during extraction of the lipid hydroperoxide.

Scheme 2: Proposed Mechanisms for (A) Oxygenation of Lipids by Lipoxygenase and (B) Interaction of Inhibitors and Lipid Hydroperoxides with Lipoxygenase under Aerobic Conditions[a]



[a] IH, inhibitor; LH, fatty acid; LOOH, fatty acid hydroperoxide; Fe, the redox-active center of the enzyme.

Since we have not been able to identify a specific oxidant in this system, we propose that the interaction of 13-HPOD with the enzyme results in the formation of the Fe^{3+} form of the enzyme, that the lipoxygenase itself is the electron acceptor, and that the 13-HPOD acts merely as a facilitator of this process. This idea is incorporated into a revised scheme for lipoxygenase activation (Scheme 2A). In this scheme we indicate that the activation of the enzyme requires 13-HPOD, which then remains bound, but chemically unchanged, during the reaction with linoleic acid. In fact we have no evidence concerning whether or not the 13-HPOD remains bound to the enzyme following activation.

One of the consequences of the proposed scheme is that the enzyme does not act as a peroxidase either during activation or under normal conditions of turnover with linoleic acid and O_2 as substrates. It has been shown by other workers that when reticulocyte lipoxygenase utilizes 15 L_S-HETE as substrate, there is a requirement for 13-HPOD to activate the enzyme and consumption of 1 mol of 13-HPOD for every 9 turnovers of the enzyme (Kuhn et al., 1986). It is not clear whether consumption of 13-HPOD occurs during the turnover of linoleic acid, but if this is the case then it is likely to be a minor pathway and would not invalidate the basic principles of Scheme 2A. This is in contrast to the circumstances when the enzyme reacts with 13-HPOD and an inibitor such as BWA4C (see Scheme 2B). Clearly, under these circumstances the 13-HPOD and the

inhibitor are both consumed and novel products are generated (Figure 7). In this case the key distinction between the inhibitor and the fatty acid substrate which it mimics is that, once oxidized, the inhibitor radical cannot be oxygenated, so allowing a kinetically slow process of reduction of the bound lipid peroxide by the ferrous iron in the active site to proceed. In this context oxygen is an inhibitor of the peroxidase reaction (cf. Scheme 2A) and this is supported by the observation that only under anaerobic conditions does linoleic acid promote significant decomposition of bound lipid peroxide by a free radical mechanism.

In summary, we show that the lipid hydroperoxide-dependent activation of lipoxygenases does not require the concomitant oxidation of the peroxide, and we propose that this function is undertaken by another group within the protein. A plausible candidate for this group may be pyrroloquinoline quinone (PQQ), which has been reported to be an organic cofactor in soybean lipoxygenase and which can stabilize an anionic radical by protonation to form PQQH$^{\bullet}$ (van der Meer & Duine, 1988). Eventual deactivation of the enzyme (see Scheme 2B) would then result from reformation of the PQQ/Fe^{2+} system in the absence of LOOH. However, Michaud−Soret et al. (1990) report that active soybean lipoxygenase does not contain PQQ. Should this indeed be the case, then an as-yet unidentified organic cofactor or amino acid residue must perform an identical function.

If the mechanism suggested by us is employed within the cell, it would allow lipid hydroperoxide levels to modulate the activity of these important enzymes without the formation of pro-oxidant lipid-derived radicals which are capable of promoting peroxidative reactions.

REFERENCES

Axelrod, B., Cheesebrough, T. M., & Laasko, S. (1981) *Methods Enzymol. 71*, 441−451.

De Groot, J. J. M. C., Veldink, G. A., Bliegenthart, J. F. G., Boldingh, J., Wever, R., & Van Gelder, B. F. (1975) *Biochim. Biophys. Acta 377*,, 71−79.

Egmond, M. R., Veldink, G. A., Bliegenthart, J. F. G., & Boldingh, J. (1973) *Biochem. Biophys. Res. Commun. 54*, 1178−1184.

Egmond, M. R., Finazzi-Agrò, A., Fasella, P. M., Beldink, G. A., & Vliegenthart, J. F. G. (1975) *Biochim. Biophys. Acta 397*, 43−49.

Egmond, M. R., Brunori, M., & Fasella, P. M. (1976) *Eur. J. Biochem. 61*, 93−100.

Egmond, M. R., Fasella, P. M., Veldink, G. A., Vliegenthart, J. F. G., & Boldingh, J. (1977) *Eur. J. Biochem. 76*, 469−479.

Falgueyret, J.-P., Hutchinson, J. H., & Riendeau, D. (1993) *Biochem. Pharmacol. 45*, 978−981.

Finazzi-Agrò, A., Aviglaino, L., Veldink, G. A., Vliegenthart, J. F. G., & Boldingh, J. (1973) *Biochim. Biophys. Acta 326*, 462−470.

Ford-Hutchinson, A. W. (1991) *Eicosanoids 4*, 65−74.

Kemal, C., Louis-Flamberg, P., Krupinski-Olsen, R., & Shorter, A. L. (1987) *Biochemistry 26*, 7064−7072.

Kühn, H., Wiesner, R., Stender, H., Schewe, T., Lankin, V. Z., & Rapoport, S. M. (1986) *FEBS Lett. 203*, 247−252.

Kühn, H., Belkner, J., Weisner, R., & Brash, A. R. (1990) *J. Biol. Chem. 265*, 18351−18361.

Ludwig, P., Holzhütter, H.-G., Colosimo, A., Silvestrini, M. C., Schewe, T., & Rapoport, S. M. (1987) *Eur. J. Biochem. 168*, 325−337.

Mansuy, D., Cucurou, C., Biatry, B., & Battioni, J. P. (1988) *Biochem. Biophys. Res. Commun. 151*, 339−346.

Michaud-Soret, I., Daniel, R., Chopard, C., Mansuy, D., Cucurou, C., Ullrich, V., & Chottard, J. C. (1990) *Biochem. Biophys. Res. Commun. 172*, 1122−1128.

Nelson, M. J., Batt, D. G. Thompson, J. S., & Wright, S. W. (1991) *J. Biol. Chem. 266*, 8225−8229.

Pistorius, E. K., & Axelrod, B. (1976) *J. Biol. Chem. 251*, 7144−7148.

Rapoport, S. M., Schewe, T., Wiesner, R., Halangk, W., Ludwig, P., Janicke-Höhne, M., Tannert, C., Hiebsch, C., & Klatt, D. (1979) *Eur. J. Biochem. 96*, 545−561. .

Reynolds, C. H. (1988) *Biochem. Pharmacol. 37*, (23), 4531−4537.

Salmon, J. (1986) *Adv. Drug Res. 15*, 111−167.

Schewe, T., Rapoport, S. M., & Kühn, H. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 191−273.

Schilstra, M. J., Veldink, G. A., Verhagen, J., & Vliegenthart, J. F. G. (1992) *Biochemistry 31*, 7692−7699.

Schilstra, M. J., Veldink, G. A., & Vliegenthart, F. G. (1994) *Biochemistry 33*, 3974−3979.

Smith, W. L., & Lands, W. E. M. (1972) *J. Biol. Chem. 247*, 1038−1047.

Van der Meer, R. A. & Duine, J. A. (1988) *FEBS Lett. 235*, 194−200.

Veldink, G. A., Vliegenthart, J. F. G., & Boldingh, J. (1977) *Prog. Chem. Fats Other Lipids 15*, 131−166.

Wang, Z.-X., Killilea, S. D., & Srivastava, D. K. (1993) *Biochemistry 32*, 1500−1509.

Wilcox, A. L., & Marnett, L. J. (1993) *Chem. Res. Toxicol. 6*, 413−416.

Yamamoto, S. (1992) *Biochim. Biophys. Acta* 1128, 117−131.

BI952425H