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Activity of Soybean Lipoxygenase in the Absence of Lipid Hydroperoxide†

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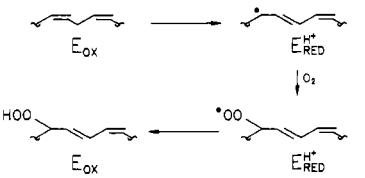
ABSTRACT: Soybean lipoxygenase was assayed under conditions such that the concentration of the enzyme was in excess of the concentration of the substrate, arachidonic acid. Under these conditions, the concentration of lipid hydroperoxides present as contaminants in the substrate was negligible relative to the enzyme concentration, and the concentration of lipid hydroperoxide product could be determined accurately. The ferric form of the enzyme was observed to be fully active and to catalyze the oxidation of arachidonic acid at a near-diffusion-controlled rate, 1.4×10^7 M^{-1} s^{-1} at 0 °C, at concentrations of lipid hydroperoxides as low as 5% of the enzyme concentration. From this, it can be concluded that the higher oxidation states that would be accessible by oxidation of Fe(III) by hydroperoxide are not required for catalysis by soybean lipoxygenase. Surprisingly, the activation of the ferrous form of the enzyme was also observed at insignificantly low lipid hydroperoxide concentrations. This activation presumably involves oxidation of the ferrous to the ferric form of the enzyme and must be more facile than has hitherto been reported. This result may rationalize previous reports that the ferrous and the ferric forms of the enzyme are both active.

The kinetics and mechanism of soybean lipoxygenase have been extensively studied, and the available information has been collected in several recent reviews (Ludwig et al., 1987; Petersson et al., 1987; Schewe et al., 1986; Kühn et al., 1986a; Lands, 1984; Veldink et al., 1984). The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron–sulfur center. The iron is considered to be the oxidant that oxidizes the 1,4-diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate (Scheme I). The pentadienyl radical is then trapped by oxygen to give a lipid hydroperoxide as the final product. For example, when the substrate is arachidonic acid as in this study, the product is 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (15-HPETE).[1] The hydroperoxide product appears to play a key role in this reaction. The iron in the isolated enzyme is almost entirely in the ferrous (Fe^{II}) state and is oxidized by lipid hydroperoxides to the ferric (Fe^{III}) state. This oxidation to the ferric form is generally considered to be necessary for enzyme activity. Several careful investigations have shown that the distinction between the ferrous and the ferric forms of the enzyme cannot always be made, however. The ferric enzyme can exhibit a lag period comparable to that of the ferrous enzyme (de Groot et al., 1975a), which suggests that the ferric form still requires lipid hydroperoxide for activity. In addition, it has been demonstrated that the reticulocyte lipoxygenase has a continuous requirement for lipid hydroperoxide during turnover (Kühn et al., 1986b). On the basis of these observations, it has been proposed that the iron in the active form of the enzyme may be in a higher oxidation state, for example, ferryl iron as in the cytochrome P-450's (Ludwig et al., 1987). Spectral evidence for these higher oxidation states as intermediates has been provided for prostaglandin H synthase, which catalyzes a similar reaction but which is a hemoprotein (Lambier et al., 1985; Kulmacz et al., 1987).

Scheme I



A major impediment to clarifying the role of lipid hydroperoxide in the lipoxygenase reaction is the fact that the unsaturated fatty acid substrates are susceptible to autoxidation. One must expect, therefore, that the substrate would invariably be contaminated with lipid hydroperoxide, perhaps at immeasurably low concentrations that would nonetheless be significant when compared to the enzyme concentration. In order to avoid this complication, we have investigated the activity of soybean lipoxygenase under conditions such that the enzyme concentration is in excess of the substrate and the concentration of lipid hydroperoxides is demonstrably insignificant.

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[1] Abbreviations: 15-HPETE, 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13-(*E*)-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13-(*E*)-eicosatetraenoic acid; HPLC, high-pressure liquid chromatography.

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Materials and Methods

[^{3}H]- and [^{14}C]arachidonic acid were from New England Nuclear, 240 Ci/mmol and 50 mCi/mmol, respectively, and unlabeled arachidonic acid was from NuCheck Prep. Arachidonic acid was purified by HPLC on silica gel with 0.1% acetic acid in hexane as eluant and stored in ethanol at 0.1 mCi/mL (labeled) or 10 mM (unlabeled) at −80 °C. The radiochemical purity of the labeled substrate was 99% or greater. The L-1 isozyme of soybean lipoxygenase was purified as described (Axelrod et al., 1981) and its purity checked by polyacrylamide gel electrophoresis in sodium dodecyl sulfate as well as by ion-exchange chromatography on a Mono Q column (Pharmacia) with a 10-mL linear gradient from 0.1 to 0.3 M NaCl in 0.1 M Tris–acetate, pH 8.0 (Ramadoss & Axelrod, 1982). The purified enzyme was shown to be at least 95% in the ferrous form by ESR spectroscopy by comparison of the signal at $g = 6.1$ for the native enzyme vs enzyme oxidized with 1 equiv of 13-hydroperoxy-9(Z),11(E)-octadecadienoic acid (Slappendel et al., 1982; Cheesbrough & Axelrod, 1983). Lipoxygenase concentrations are based on M_r 94 000 and $A_{280\,nm}^{0.1\%} = 1.6$ (Petersson et al., 1987; Shibata et al., 1987). Bovine erythrocyte glutathione peroxidase was from Calbiochem; the activity of this enzyme is expressed in the supplier's units.

Lipoxygenase assays were performed at 0 °C in 0.05 M sodium borate at pH 9.0 or 9.5 as indicated. Substrate was added as a solution in ethanol so that the assay solution was 0.2% in ethanol. The pH optimum is 9.0. The critical micelle concentration for arachidonic acid was shown to be greater than 0.1 mM under these conditions. Aliquots, 0.5 mL, of the reaction mixture were quenched by addition to 0.5 mL of a solution at 0 °C consisting of 750:300:12 $CH_3CN/H_2O/H_3PO_4$ and containing unlabeled arachidonic acid at 20 μg/mL. It was essential to include the unlabeled arachidonic acid and to cool the quench solution to 0 °C in order to minimize decomposition of the 15-HPETE product. Product formation was assayed by HPLC (Skoog et al., 1986), as was reduction of 15-HPETE to 15-HETE in assays containing glutathione peroxidase (Hogaboom et al., 1986). Reactions at >3 nM enzyme were performed similarly in a rapid quench apparatus (Hi-Tech Scientific) except that unlabeled arachidonic acid was added after the reaction mixture was quenched to avoid contaminating the apparatus with high arachidonic acid concentrations. The apparatus was washed extensively with acetonitrile after each reaction. The reaction rates measured in the rapid quench apparatus were consistently approximately 70% of the rates predicted by extrapolation from lower enzyme concentrations. This difference can be ascribed to artifacts such as binding to the vessel walls and dilution in the reaction loop.

Ferric lipoxygenase was prepared by incubating 0.4 mL of 3.5 μM enzyme in borate buffer, pH 9.0, with 10 μM [^{14}C]-arachidonic acid for 3 min at 0 °C (Slapendel et al., 1982). The reaction mixture was chromatographed on Sephadex G50 (fine, 0.7 × 20 cm) in borate buffer plus 0.2 M NaCl, and the protein-containing fractions were pooled and assayed for enzymatic activity and radioactivity. Recovery of protein was typically 50% without a decrease in specific activity of the enzyme. A sample of ferrous lipoxygenase for comparison was prepared identically but omitting the incubation with arachidonic acid. The enzyme was used within 30 min of elution from the column.

Results

In order to determine whether lipid hydroperoxides are required for the soybean lipoxygenase reaction, the ferrous enzyme was first converted to the ferric form by incubation with 3 equiv of [^{14}C]arachidonic acid. The enzyme was then isolated by gel chromatography of the reaction mixture. It was demonstrated that only 0.016 equiv of fatty acid remained bound to the enzyme; i.e., enzyme was separated from essentially all lipid hydroperoxide. The ferric enzyme was assayed by using a very low substrate concentration (0.8 nM) so that the lipid hydroperoxide product generated would be less than the enzyme concentration (1.3 nM) (Figure 1A). Since the concentration of the substrate was well below its K_m, oxidation would be expected to be first order in substrate, and this was observed. There was no lag for the ferric enzyme. The observed first-order rate constant divided by the enzyme concentration gives $k_{cat}/K_m = 1.8 \times 10^7\ M^{-1}\ s^{-1}$. This rate constant is very near the diffusion-controlled limit of $6 \times 10^7\ M^{-1}\ s^{-1}$ at 0 °C. Furthermore, it is equal to the maximum value of k_{cat}/K_m that we have observed for pure lipoxygenase. In a series of experiments, this value for k_{cat}/K_m was constant within the range 0.6–150 nM enzyme and 3 nM to 2 μM substrate (K_m is approximately 10 μM). It is unlikely, therefore, that the activity observed for the ferric enzyme is due only to a small active fraction of the total enzyme but rather due to fully active enzyme. If care was taken to prevent decomposition of the product during analysis, then 15-HPETE accounted for 80–90% of the products so that there was no indication that the hydroperoxide that was formed reacted further with the ferric enzyme.

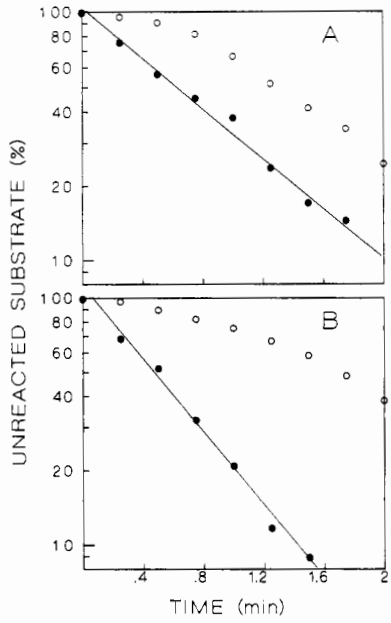

FIGURE 1: Comparison of ferric and ferrous lipoxygenase activities at low substrate concentrations. (A) Lipoxygenase, 1.3 nM, was incubated with 0.8 nM [^{3}H]arachidonic acid at 0 °C in borate buffer at pH 9.0. (B) Assays performed similarly but in the presence of 1 mM glutathione and glutathione peroxidase, 6 units/mL. (○) Ferrous lipoxygenase. (●) Ferric lipoxygenase. The apparent small lag observed for the ferric enzyme is due to the time required to mix substrate and enzyme.

The ferric enzyme was also assayed in the presence of glutathione peroxidase, which further lowered the concentration of 15-HPETE in the assay by reducing it to the alcohol, 15-HETE. The relative concentrations of the alcohol and the hydroperoxide could be measured by HPLC, and the level of 15-HPETE was found to be less than 5% of the total product at the enzyme concentration used. The results demonstrate that the ferric lipoxygenase is fully active even at these very low peroxide concentrations (Figure 1B).

In contrast, the ferrous enzyme was initially not active upon

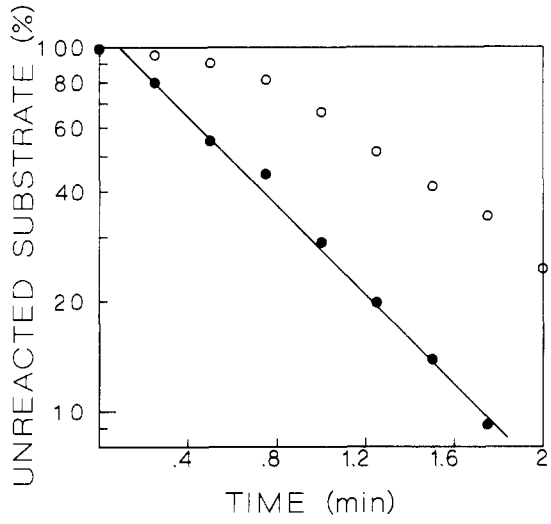

FIGURE 2: Autoactivation of ferrous lipoxygenase. Ferrous lipoxygenase, 1.3 nM, was incubated with 0.8 nM [^{3}H]arachidonic acid at 0 °C, pH 9.0. (○) Enzyme was diluted 1000-fold into a solution containing substrate to initiate the assay. (●) Enzyme was diluted 1000-fold with buffer, and substrate was added 3 min later to initiate the assay.

addition of substrate. For example, a sample of the Fe^{II} form of the enzyme was treated in parallel with the ferric enzyme as described above, except that the oxidation by incubation with arachidonic acid was omitted. The ferrous enzyme shows a clear lag that is extended in the presence of glutathione peroxidase (Figure 1). Such a lag is characteristic of the ferrous lipoxygenase and has been ascribed to the autocatalytic activation of the Fe^{II} enzyme by oxidation by the hydroperoxide product (de Groot et al., 1975b). This cannot be the explanation in the present case, however, since there was insufficient lipid hydroperoxide formed to account for the activation of the enzyme, and there is no evidence that the peroxide that was formed reacted with the enzyme. Thus, in the absence of glutathione peroxidase, full activity was achieved by 1 min, at which time only 0.3 nM arachidonic acid had been consumed by 1.3 nM enzyme, and 80–90% of the consumed arachidonic acid could be accounted for as 15-HPETE. In addition, in the presence of glutathione peroxidase, half-maximal activity was achieved by 2 min, when the 15-HPETE available was less than 5% of the enzyme concentration. These results imply that the activation of soybean lipoxygenase does not require lipid hydroperoxide. This was confirmed by diluting enzyme to the normal assay concentration and initiating an assay by addition of substrate 5 min later (Figure 2). The enzyme was fully active after this short incubation.

The apparent autoactivation of the ferrous enzyme was observed under a variety of conditions. As implied by the results in Figure 1, activation is slower in the presence of glutathione peroxidase. It was also slowed by horseradish peroxidase (20 units/mL, 0.2 mM phenol) and catalase (2000 units/mL). The rate of autoactivation was highly variable, however, as was the retardative effect of these three enzymes. The rate of activation was also faster in polypropylene test tubes than in glass and in some types of polypropylene faster than in others. These results, and particularly the variability in the rate of activation, suggest that the autoactivation is caused by impurities and possibly by impurities on the surface of the reaction vessels. Thorough washing of the reaction vessels with detergent or ethanol or soaking in 1 M sodium dithionite, a strong reducing agent, had little effect, however. The autoactivation was observed at enzyme concentrations as high as 150 nM in rapid quench experiments and at up to 0.2 mM substrate, so that it is a general phenomenon and not limited to very high dilutions of enzyme or substrate. More extensive characterization of this autoactivation of the ferrous enzyme has been frustrated by the extreme variability in the rate of activation under apparently identical conditions.

The 5-lipoxygenase from rat PMN undergoes a similar autoactivation (data not shown).

Discussion

The results presented herein have demonstrated that the Fe^{III} form of soybean lipoxygenase does not require lipid hydroperoxide for activity. The ferric enzyme catalyzes the oxidation of arachidonic acid at near-diffusion-controlled rates and without a lag under conditions such that the concentration of lipid hydroperoxides is demonstrably negligible. It follows that higher oxidation states of iron are not required for catalytic activity. This enzyme differs, therefore, in its chemistry and mechanism of catalysis from the hemoproteins such as cytochrome P-450, for which the ferryl oxidation state is normally the active oxidant. While our results are relevant to a single or a limited number of turnovers, they must be reconciled with observations that for multiple turnovers there is continuing requirement for lipid hydroperoxide (Lands et al., 1971; Kühn et al., 1986b). It is reasonable that the enzyme might lose activity over many turnovers and require lipid hydroperoxide for reactivation. This situation could arise if an intermediate occasionally dissociated while the iron was in the ferrous state.

We have also observed that the activation of ferrous lipoxygenase is more facile than has been previously reported. Thus, the enzyme can be activated within several minutes simply by dilution to concentrations below 1 μm. It is assumed that this activation represents oxidation of the ferrous to the ferric form of the enzyme. This is not proven, however, since there is currently no procedure to distinguish the two oxidation states at these low concentrations. Attempts to characterize the autoactivation reaction in detail have been frustrated by its irreproducibility, but it has the characteristics, including the irreproducibility, of a reaction dependent upon impurities in the reaction buffers or especially on the surface of the reaction vessels. The impurities may either oxidize the enzyme directly or catalyze its oxidation by atmospheric oxygen. Previous reports have offered evidence that Fe^{II} lipoxygenase is itself active (de Groot et al., 1975a; Egmond et al., 1977). The observation that the Fe^{II} enzyme is easily activated offers an alternative explanation for these results. Particularly convincing results had been obtained in stopped-flow experiments at high concentrations of ferrous lipoxygenase and relatively low concentrations of carefully purified substrate, 0.3 and 30 μM, respectively (Egmond et al., 1977). We have obtained similar results at 150 nM enzyme and 40 nM substrate in stop-quench experiments. Our own results, therefore, clearly substantiate the previous claims that the ferrous enzyme appears to be active in the absence of lipid hydroperoxide. It cannot be safely concluded, however, that the enzyme under these conditions is still in the ferrous form.

Registry No. 15-HPETE, 70981-96-3; lipoxygenase, 63551-74-6; arachidonic acid, 506-32-1.

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Inactivation of Soybean Lipoxygenase 1 by 12-Iodo-*cis*-9-octadecenoic Acid†

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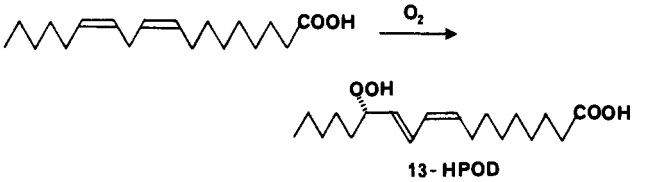
ABSTRACT: 12-Iodo-*cis*-9-octadecenoic acid (12-IODE) is a time-dependent, irreversible inactivator of soybean lipoxygenase 1. The rate of inactivation is independent of 12-IODE concentration above 20 μM and is half-maximal at about 4 μM. Inactivation by 12-IODE requires lipid hydroperoxide, which must be present even after the initial oxidation of the iron in the enzyme from ferrous to ferric. Inactivation by 12-IODE is also dependent on O_2. These findings suggest that 12-IODE is converted by the enzyme into a more reactive species, which is responsible for inactivation. No inactivation has been detected with 12-iodooctadecanoic acid, 12-bromo-*cis*-9-octadecenoic acid, 12-iodo-*trans*-9-octadecenoic acid, or a mixture of stereoisomers of 9,11-octadecadienoic acid.

Lipoxygenases catalyze the oxygenation of *cis,cis*-1,4-dienes to produce conjugated diene hydroperoxides. Enzymes of this type are widespread in the plant kingdom and have recently been found to be important in mammalian arachidonic acid metabolism (Needleman et al., 1986). One of the mammalian enzymes is a target for drug design, since it is involved in the biosynthesis of leukotrienes, which are produced in inflammation and anaphalaxis (Hammarström, 1983). Since the mammalian enzymes are difficult to purify, attempts to design mechanism-based inhibitors rely heavily on structural and mechanistic studies on the plant enzymes, particularly lipoxygenase 1 from soybeans (Veldink et al., 1977; Veldink & Vliegenthart, 1984). This enzyme is specific for fatty acids in which the 1,4-diene unit begins on the ω6 carbon, that is, the sixth carbon from the methyl terminus (Hamberg & Samuelsson, 1967; Holman et al., 1969). For example, the enzyme catalyzes the conversion of linoleic acid to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD):[1]



13-HPOD

Lipoxygenase 1 consists of a single polypeptide chain (M_r 94 500), the sequence of which has recently been determined (Shibata et al., 1987). The enzyme contains one atom of non-heme iron per molecule (Chan, 1973; Roza & Francke, 1973; Pistorius & Axelrod, 1973), which appears to be essential

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[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 13-HOD, 13(*S*)-hydroxy-*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; methyl 12-MsODE, methyl 12-[(methylsulfonyl)oxy]-*cis*-9-octadecenoate; GC/MS, gas chromatography/mass spectrometry; MS, mass spectrometry; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; IR, infrared; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.

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