Secondary Alkyl Hydroperoxides as Inhibitors and Alternate Substrates for Lipoxygenase†

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ABSTRACT: Lipoxygenase plays a central role in polyunsaturated fatty acid metabolism, inaugurating the biosynthesis of eicosanoids in animals and phytooxylipins in plants. Redox cycling of the non-heme iron cofactor represents a critical element of the catalytic mechanism. Paradoxically, the isolated enzyme contains Fe(II), but the catalytically active form contains Fe(III), and the natural oxidant for the iron is the hydroperoxide product of the catalyzed reaction. Controlling the redox state of lipoxygenase iron with small molecules, inhibitors or activators, could be a means to modulate the activity of the enzyme. The effects of secondary alkyl hydroperoxides and the corresponding alcohols on soybean lipoxygenase-1 reaction rates were investigated and found to be very different. Secondary alcohols were noncompetitive or linear mixed inhibitors with inhibition constants in the millimolar concentration range, with more hydrophobic compounds producing lower values. Secondary alkyl hydroperoxides were inhibitors of lipoxygenase-1 primarily at high substrate concentration. They were more effective inhibitors than the alcohols, with dissociation constants in the micromolar concentration range. The hydroperoxides bearing longer alkyl substituents were the more effective inhibitors. Oxidation of the iron in lipoxygenase-1 by 2-hydroperoxyalkanes was evident in electron paramagnetic resonance (EPR) measurements, but the enzyme was neither activated nor was it inactivated. Instead there was evidence for an entirely different reaction catalyzed by the enzyme, a homolytic dehydration of the hydroperoxide to produce the corresponding carbonyl compound.

In animals, polyunsaturated fatty acid metabolism is governed by two enzymes: cyclooxygenase and lipoxygenase (*1*). These enzymes initiate the conversion of arachidonic acid into families of compounds with important biological properties known collectively as eicosanoids. Cyclooxygenase catalysis converts arachidonic acid into the prostaglandins, compounds with mostly proinflammatory properties. In a similar fashion, lipoxygenases convert arachidonic acid into leukotrienes, hepoxylins, lipoxins, and HETEs.[1] The products of arachidonic acid metabolism play important roles in human health and disease (*2*, *3*). Lipoxygenases also mediate polyunsaturated fatty acid metabolism in plants (*4*). The products, which are derived from linoleic and linolenic acids, are referred to as phytooxylipins and include jasmonic acid, traumatin, and volatile aldehydes. These compounds are particularly important in the plant wound response and also more generally in germination, development, and growth.

Lipoxygenases are large monomeric proteins that possess a single non-heme iron cofactor (*5*). Redox cycling of the iron between the 3+ and 2+ states is a critical feature of the mechanism of the catalyzed reaction (*6*). While isolated lipoxygenases contain iron(II), the starting point for the catalytic cycle is iron(III). Oxidation of the iron takes place through a reaction with the hydroperoxide product of the catalyzed reaction, 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, 13-HPOD (Scheme 1) (*7*). Manipulating the oxidation state of lipoxygenase iron could provide an opportunity to control the activity of the enzyme for both activation (oxidation) and inhibition (reduction). There are classes of lipoxygenase inhibitors specifically designed to bind to and/or reduce the iron atom (*8*). Metal chelating compounds, however, tend not to display particularly high selectivity, as they form complexes with a wide variety of free and protein-bound metals. We have inaugurated a program of research to better understand the activation chemistry of lipoxygenases and to identify small molecules that might selectively influence the redox reactions.

The first compound we investigated was cumene hydroperoxide, which had a complex set of effects on soybean lipoxygenase-1 and -3 (*9*). The hydroperoxide was an inhibitor of the maximum (steady-state) rate of the lipoxygenase-1-catalyzed oxygenation of linoleic acid at pH 9, but only at high substrate concentration. This finding identified a potential small molecule binding site on the enzyme−substrate complex and the ensuing inhibition. The iron in lipoxygenase was oxidized by cumene hydroperoxide. The iron(III) form of the enzyme was readily identified by EPR spectroscopy. However, preincubation with the peroxide led to almost complete inactivation with respect to catalysis, not activation. The cause of the peroxide-induced inactivation

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[1] Abbreviations: HETE, hydroxyeicosatetraenoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; EPR, electron paramagnetic resonance; SCE, saturated calomel electrode.

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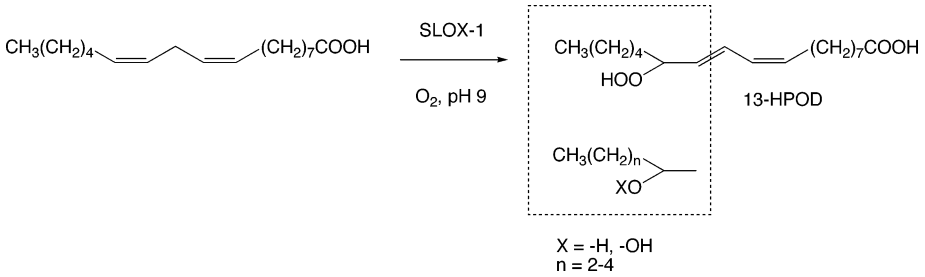
Scheme 1



was traced to the β-hydroxylation of one of two active-site amino acids, Trp-519 and Leu-565 (lipoxygenase-3 numbering). In seeking to explore the scope and generality of the chemistry obtained with cumene hydroperoxide, we have investigated the effects of secondary alkyl hydroperoxides and alcohols on lipoxygenase catalysis, compounds that mimic the ω-end of the peroxide product (Scheme 1).

The two classes of compounds, differing only by the presence or absence of an oxygen atom, had significantly different consequences for lipoxygenase catalysis.

EXPERIMENTAL PROCEDURES

Materials. Soybean lipoxygenase-1was obtained from seeds (cv. Resnik) by extraction, ammonium sulfate fractionation, and chromatofocusing (*10*). The secondary alcohols were obtained from Aldrich. The hydroperoxides were synthesized by autoxidation of Grignard reagents (*11*), which were prepared from the corresponding chloroalkanes. The chloroalkanes were obtained from Aldrich or were made from the alcohol by reaction with thionyl chloride (*12*). The hydroperoxides were purified by chromatography on silica with hexane and acetone 9:1 (v/v). Stock solutions of the compounds were prepared by weight in absolute methanol and stored at −80 °C. 13-HPOD was prepared from linoleic acid by a previously reported procedure (*13*). Samples of 13-HPOD were prepared in absolute methanol and stored at −80 °C. The concentration was determined by dilution in absolute methanol and UV−vis spectrophotometry with an ϵ value of 23 000 L/mol·cm.

Methods. The maximum rate of the lipoxygenase-catalyzed reaction with linoleic acid as substrate was determined spectrophotometrically at 234 nm in 0.1 M borate buffer, pH 9.0, at 25 °C as previously described (*9*). All solutions contained 2% methanol (v/v). The reproducibility of the rate measurements both serial and occasional was within 10% (shown). The rates of the reactions incorporating secondary alcohols were evaluated with the program Enzyme Kinetics!Pro (version 2.36, SynexChem, LLC). The maximum rate data for the reactions conducted in the presence of the hydroperoxides were simulated with the interactive program DynaFit (*14*). The mechanism of Schilstra et al. (*15*) was employed with rate/dissociation constants of $K_P = K_P^* = 12\ \mu M$, $K_S = K_S^* = 12\ \mu M$, $k_1 = 300\ s^{-1}$, $k_2 = 10^9\ M^{-1}\ s^{-1}$, $k_3 = 2300\ s^{-1}$, and $k_4 = 150\ s^{-1}$. Preincubation experiments were conducted in 0.1 M borate buffer, pH 9.0, at 25 °C with lipoxygenase-1 at 1.1 μM and the hydroperoxides at the indicated ratios. Aliquots were removed at 15, 30, and 45 min for enzymatic assay. The reported values reflect the average of triplicate analyses at each time point and the average for the three time points.

EPR spectroscopy was carried out at 9 GHz on a Bruker Model ESP 300E spectrometer equipped with an Oxford Instruments Model ITC4 cryostat operating at 25 K. The microwave power was 5 mW with a modulation amplitude of 1 mT. The secondary alkyl hydroperoxides were combined with lipoxygenase-1 (200 μM final concentration) in 0.1 M Tris-HCl buffer at pH 7.0 at 0 °C by direct injection of a stock solution in methanol. The final volume of methanol in the EPR samples was 2% (v/v). The samples were incubated on ice for 10 min and then transferred to EPR tubes and frozen in liquid nitrogen.

Isothermal titration calorimetry experiments were carried out at 30 °C in a MicroCal VP-ITC microcalorimeter. A degassed solution of the enzyme in Tris-HCl (0.1 M, pH 7.0) was placed in the cell. The concentration of the enzyme was determined spectrophotometrically at 280 nm, with a value of 120 000 L/mol·cm for ϵ (*16*). A degassed solution of 2-heptanol or 2-hydroperoxyheptane in exactly the same buffer was placed in the syringe. The titration consisted of 30 injections of 0.010 mL. When the same solution of alcohol or peroxide was injected into the buffer, a consistent amount of heat small enough to be negligible was evolved.

The reaction product was analyzed on a Hewlett-Packard model 5890 series II gas chromatograph with a 5971 series mass-selective detector. The chromatography was carried out on a HP-5 (30 m × 0.32 mm) column with a linear temperature program from 50 to 200 °C. 2-Hydroperoxyheptane was treated with lipoxygenase-1 (2.0 mL, 2.55 mg/mL, 0.054 μmol) in a 10:1 molar ratio for 30 min at room temperature. The product was isolated by drawing the mixture through a preconditioned solid-phase extractor (Thermo Hypersep C18, 500 mg, 6 mL). The extractor was washed with water (3×, 2 mL), and the product was eluted in methylene chloride (2 mL). Aliquots (10 μL) of the methylene chloride extracts were analyzed directly by GCMS. 2-Hydroperoxyheptane was subjected to the same procedure without the enzyme for the control. A small amount of thermal decomposition of the hydroperoxide into the alcohol and ketone was evident in the control, with a retention time around 4 min, but not in the enzyme-treated sample.

The molecular graphics image was created with VMD (*17*) and rendered with POV Ray.

RESULTS

The 2-alkanols, 2-pentanol, 2-hexanol, and 2-heptanol were all inhibitors of the maximum rate of lipoxygenase-1 catalysis at pH 9. 2-Heptanol had the characteristics of a noncompetitive inhibitor, while 2-hexanol and 2-pentanol gave rate effects that were typical for linear mixed-type

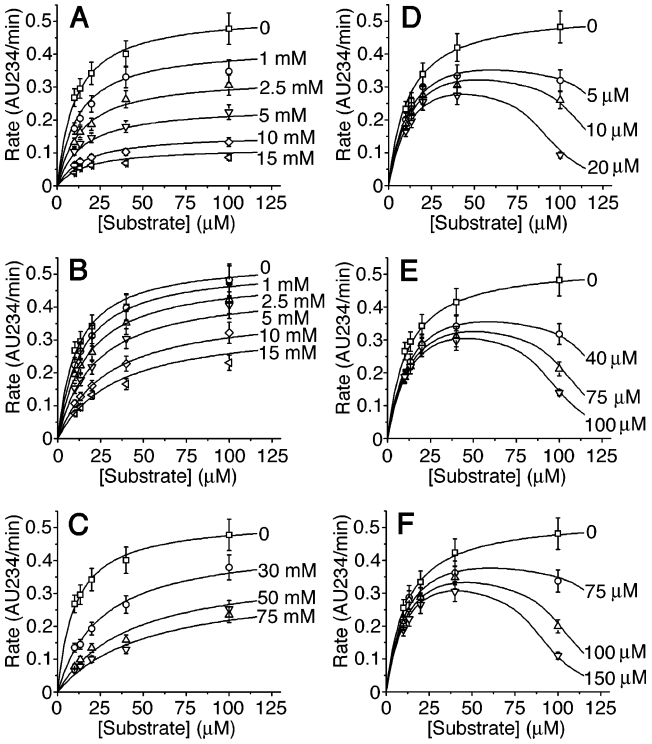


FIGURE 1: Effect of different concentrations (indicated) of secondary alcohols (A−C) and secondary hydroperoxides (D−F) on the maximum rate of lipoxyenase catalysis over a range of substrate (linoleic acid) concentrations, in 0.1 M borate buffer, pH 9.0, at 25 °C: (A) 2-heptanol, (B) 2-hexanol, (C) 2-pentanol, (D) 2-hydroperoxyheptane, (E) 2-hydroperoxyhexane, and (F) 2-hydroperoxypentane.

Table 1: Inhibition Constants

	K_i (mM)	K_i' (mM)
2-heptanol	3.8 ± 0.2	
2-hexanol	4.6 ± 0.3	18
2-pentanol	13 ± 2	125
	K_{hp} (μM)	K_{ahp} (μM)
2-hydroperoxyheptane	12 ± 2	22
2-hydroperoxyhexane	78 ± 5	140
2-hydroperoxypentane	134 ± 24	473

inhibition (Figure 1 and Table 1). 2-Heptanol was the most effective of the alcohols with respect to inhibition of lipoxygenase-1, and 2-pentanol was the least effective. 2-Heptanol binding was also observed by isothermal titration calorimetry (Figure 2). The parameters derived from the titration were N, 0.99 ± 0.13, ΔH, −5.3 ± 0.7 kcal/mol, and ΔS, −4.1cal/deg·mol. The reciprocal of the equilibrium constant was 1.3 mM. Since inhibition could depend on multiple factors and binding was relatively weak, the value was in reasonable agreement with the inhibition constant. By contrast, the 2-hydroperoxyalkanes were inhibitors of the maximum rate of the lipoxygenase-1-catalyzed reaction primarily at high substrate concentration (Figure 1, Table 1), the same behavior observed previously for cumene hydroperoxide. The rate data for the hydroperoxides were fit to the mechanism of Schilstra et al. (*15*) modified to include 2-hydroperoxide binding to both the free and substrate-bound forms of the enzyme (Scheme 2). Least-squares optimization by use of the program Dynamax resulted in calculated values for dissociation constants for

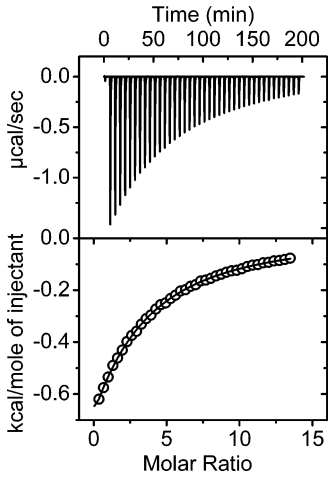
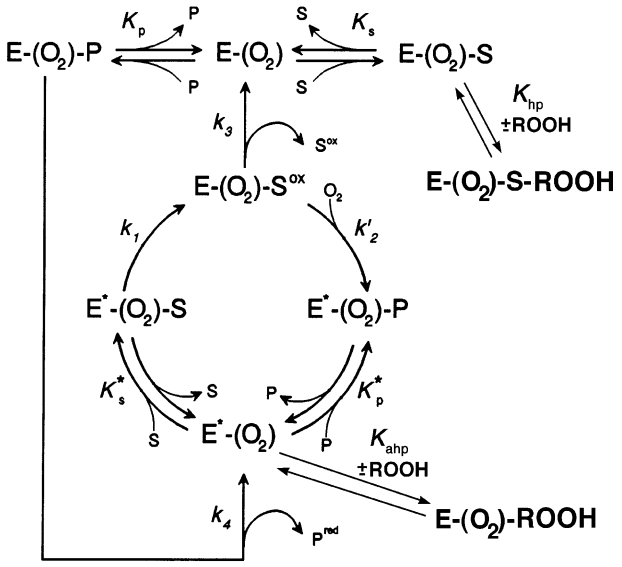


FIGURE 2: Isothermal titration calorimetry for the interaction between 2-heptanol and lipoxygenase-1 at 30 °C. Lipoxygenase-1 (0.179 mM, in 0.1 M Tris-HCl buffer, pH 8.5) was in the cell, and 0.010 mL injections of 2-heptanol (11.5 mM, matching buffer) were delivered from the syringe.

Scheme 2



the peroxides (Table 1). The hydroperoxides were more than 2 orders of magnitude more effective inhibitors compared with the alcohols, but only at high substrate concentration. As was found for the alcohols, the hydroperoxide with the longest alkyl substituent was the most effective inhibitor.

EPR spectroscopy was carried out on samples of lipoxygenase-1 treated with varying ratios of the 2-hydroperoxyalkanes to determine if the compounds oxidized the cofactor iron as was observed for 13-HPOD and cumene hydroperoxide. Slappendel et al. (*18*) showed that the EPR signals for 13-HPOD-treated lipoxygenase-1 were sensitive to the presence of low molecular weight alcohols. This has been illustrated for comparison purposes for methanol incorporation in Figure 3B. In the absence of methanol, the treatment of lipoxygenase-1 with a molar equivalent amount of 13-HPOD produced a two-part $g = 6$ signal, consistent with literature precedent (Figure 3B, center). Treatment with more than 1 molar equiv of 13-HPOD produced a spectrum dominated by a signal at $g = 4.3$ (Figure 3B, right). In the presence of methanol (2% in the EPR sample, v/v), a homogeneous signal at $g = 6$ was obtained for treatment of

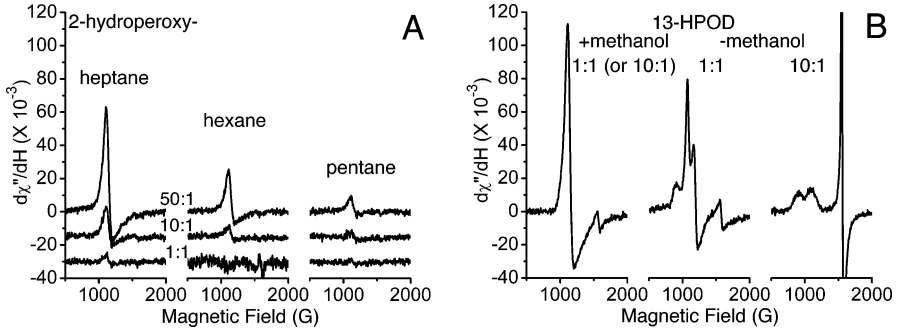


FIGURE 3: EPR spectroscopy (9 GHz) for lipoxygenase-1 treated with secondary alkyl hydroperoxides: (A) lipoxygenase-1 (Tris-HCl, pH 7.0, 10 min, 0 °C) treated with the hydroperoxides in various molar ratios (spectra offset for clarity) in the presence of 2% (v/v) methanol; (B) lipoxygenase-1 treated with 13-HPOD in the presence (left) and absence (right) of 2% (v/v) methanol; same scale as for panel A. Spectral conditions: microwave power 5 mW; field modulation 1 mT; temperature 25 K.

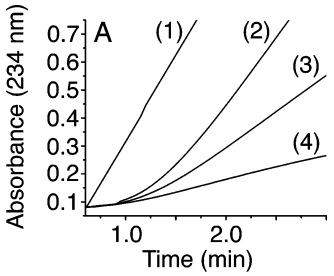


FIGURE 4: Comparison of the effects of 2-hydroperoxyheptane and 13-HPOD on the initial portion of the progress curve for lipoxygenase-1-catalyzed oxygenation of linoleic acid (100 μM, pH 9.0, in 0.1 M borate buffer, 25 °C). The effect was instantaneous when the peroxides were combined individually with the substrate. The reaction was initiated by addition of enzyme. (1) 13-HPOD, 30 μM; (2) no hydroperoxide added; (3) 2-hydroperoxyheptane, 10 μM; (4) 2-hydroperoxyheptane, 20 μM; each line is the average of three determinations.

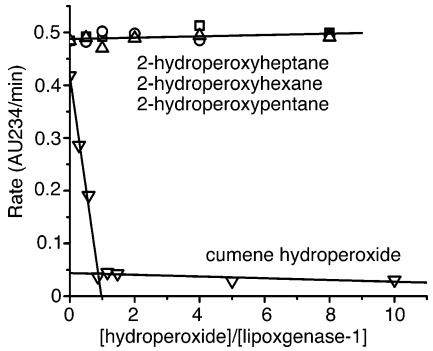


FIGURE 5: Preincubation of hydroperoxides at the indicated molar ratios for 15, 30, and 45 min at room temperature followed by maximum rate determination (100 μM linoleic acid, pH 9.0, in 0.1 M borate buffer, 25 °C). The lines represent linear least-squares fits of data points.

lipoxygenase-1 with either an equivalent or an excess amount of 13-HPOD (Figure 3B, left). These results were all consistent with the observations of Slappendel et al. (*18*). The EPR spectra for samples of lipoxygenase-1 treated with 2-hydroperoxyalkanes were obtained under the same conditions and plotted on the same scale as for the 13-HPOD-treated samples (Figure 3A). The samples were prepared by direct injection of the hydroperoxide as a methanol solution (2% in the EPR sample, v/v) into a solution of lipoxygenase-1. All samples were incubated for 10 min on ice and frozen in liquid nitrogen for the EPR measurements, which were conducted at 25 K. Each of the three hydroperoxides partially oxidized the cofactor iron in a concentration-dependent fashion. 2-Hydroperoxyheptane produced the most iron(III) as reflected in the intensity of the $g = 6$ EPR signals, followed in order by 2-hydroperoxyhexane and 2-hydro peroxypentane.

The 2-hydroperoxyalkanes did not abolish the lag phase of the time course for lipoxygenase-1 catalysis, an instantaneous effect of 13-HPOD. This is illustrated in Figure 4. The characteristic lag-phase portion of lipoxygenase-1 progress curves was completely abolished by incorporation of 13-HPOD at 30 μM with 100 μM linoleic acid as substrate when the reaction was initiated by addition of enzyme. By contrast, there was no evidence for any reduction in the lag phase when 2-hydroperoxyheptane was combined with the substrate prior to initiation of the reaction. A small but significant reduction in the lag phase was seen when the enzyme was first preincubated with 2-hydroperoxyheptane for 15 min at room temperature, but the effect did not depend on the concentration of the hydroperoxide and was also observed when the enzyme was preincubated with 2-heptanol at various ratios (not shown). This small effect appeared to be more related to dilution than preincubation.

When lipoxygenase-1 was incubated with cumene hydroperoxide, enzyme activity was drastically reduced (*9*). This took place instantaneously and in a stoichiometric fashion. Preincubation of lipoxygenase-1 with the 2-hydroperoxyalkanes under the same conditions was not accompanied by any loss of catalytic activity. A comparison of the effect of cumene hydroperoxide and the 2-hydroperoxyalkanes is provided in Figure 5. There was further evidence for substantially different outcomes for treatment of lipoxygenase-1 with 2-hydroperoxyheptane and cumene hydroperoxide in isothermal titration calorimetry experiments (Figure 6). We previously demonstrated that cumene hydroperoxide and lipoxygenase-1 undergo a stoichiometric exothermic reaction (reproduced in Figure 6A) comparable to the one observed for 13-HPOD and the enzyme (*9*). When lipoxygenase-1 was treated under similar conditions with 2-hydroperoxyheptane, a large amount of heat was evolved, comparable to what was seen for both cumene hydroperoxide and 13-HPOD (Figure 6B). However, there was no indication for the stoichiometric titration of the enzyme, as there was no diminution in the amount of heat obtained for each subsequent injection. A comparable experiment using the same amount of enzyme, but two times the 2-hydroperoxyheptane concentration, produced the same amount of heat per mole of injectant and showed no indication of a titration

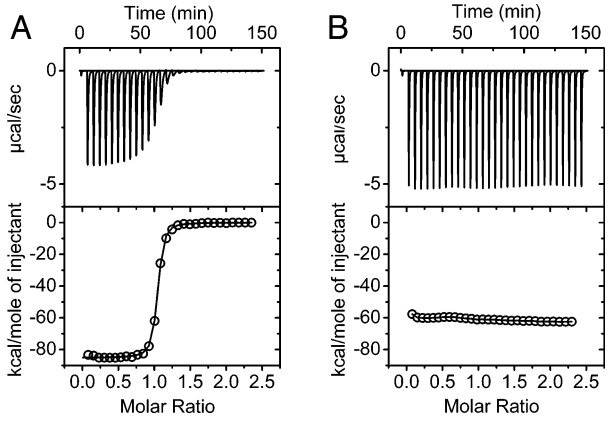


FIGURE 6: Comparison of the isothermal titration calorimetry for the interaction between lipoxygenase-1 and (A) cumene hydroperoxide or (B) 2-hydroperoxyheptane at 30 °C. Lipoxygenase-1 (0.027 mM, in 0.1 M Tris-HCl buffer, pH 7.0) was in the cell, and 0.010 mL injections of the hydroperoxides (0.277 mM, matching buffer) were delivered from the syringe.

taking place up to a 5:1 molar ratio of peroxide to enzyme (not shown). We interpret the observation of the uniform production of substantial heat in this fashion as evidence for a reaction catalyzed by the enzyme with 2-hydroperoxyheptane as the substrate. A reaction was carried out in Tris-HCl buffer at room temperature for 30 min between lipoxygenase-1 and 2-hydroperoxyheptane. The reaction mixture was applied to a solid-phase extractor, which was washed with water and eluted with dichloromethane. A single product, 2-heptanone, was detected by GC-MS analysis of samples of the dichloromethane (Figure 7).

DISCUSSION

The activating effect of 13-HPOD on lipoxygenase-1 catalysis was first described by Haining and Axelrod (*19*). The redox chemistry accompanying treatment of the enzyme by the product of its catalyzed reaction was demonstrated by the observation of EPR signals characteristic of high-spin iron(III) (*20*). The isolated enzyme contains iron(II). It was later shown that there is more to the activating effect than the oxidation of the iron atom. Kinetic studies implicated a complex mechanism involving species with both oxidation states of iron and the association of substrate and product with both (Scheme 2) (*15*). In order to gain a deeper understanding of the activating effect of 13-HPOD toward lipoxygenase catalysis, we have undertaken an investigation of the effects of other organic hydroperoxides on the enzyme. A complete understanding of the activation chemistry could be useful in devising tactics for the modulation of lipoxygenase catalysis for therapeutic purposes. Cumene hydroperoxide, for example, was found to oxidize the iron in lipoxygenase, but rather than activating the enzyme, it caused nearly complete inactivation (*9*). This was the consequence of the liberation of a reactive species, presumably a free radical, which was capable of inaugurating the β-hydroxylation of active-site amino acids. In this study, a series of secondary alkyl hydroperoxides was chosen to closely resemble a portion of the structure of 13-HPOD, the ω-6 hydroperoxide functionality.

Like cumene hydroperoxide, the secondary alkyl hydroperoxides were instantaneous inhibitors in the micromolar concentration range of lipoxygenase-1 catalysis primarily at high substrate concentration. The effect was dependent on the presence of the hydroperoxide functional group, since the corresponding alcohols did not have the same effect. The alcohols were also inhibitors, but they had the characteristics of noncompetitive or linear mixed inhibitors in the millimolar concentration range. In both cases, it was the compound with the most hydrophobic substituent that was the most effective inhibitor. There is considerable evidence for a hydrophobic binding cavity at the active site of lipoxygenase, and the observations reported here were consistent with this. Previously reported small molecule inhibitors contained hydrophobic substituents, and the extent of inhibition typically was sensitive to the length. For example, even carbon number primary alcohols were identified as competitive inhibitors of lipoxgenase-1 with K_i values ranging from 100 μM to 50 mM (*21*). The compounds with the longest chains (C8−C12) produced the lowest inhibition constants. In a study of a class of compounds referred to as half-product analogues, we showed that a hydrophobic substituent of optimal length and an electron-rich component, either −S- or C═C, constituted the structural features necessary for competitive inhibition (*22*). Studies of substrate specificity also led to the conclusion that the polyunsaturated fatty acid was bound to a hydrophobic pocket with a depth specific for correct positioning of the 1,4-pentadiene for oxygenation (*23*). Finally, in the X-ray crystallographically determined structure of the complex between lipoxygenase-3 and 13-HPOD, the -C_5H_{11} alkyl substituent of the fatty acid was found in a hydrophobic location adjacent to the cofactor iron (Figure 8) (*24*). In order to account for the kinetics data, we imagine that the 2-hydroperoxyalkanes interact with the enzyme at a similar site in lipoxygenase-1 in a substrate-dependent fashion, indicating that the active site is sufficiently spacious to accommodate two molecules simultaneously. The alcohols, with their noncompetitive kinetic effects, presumably bind elsewhere (*25*). It is remarkable that two classes of compounds differing only by the presence/absence of a single oxygen atom have such different effects on lipoxgyenase catalysis. The findings implicate a specific role for the

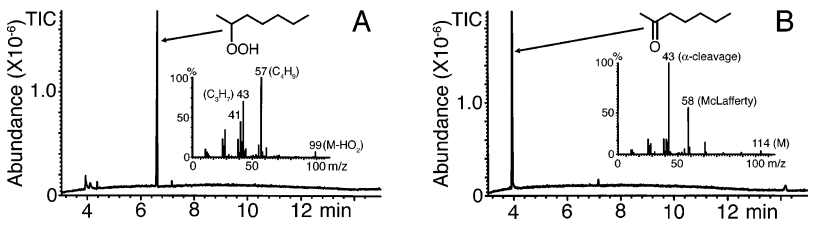


FIGURE 7: GCMS analysis of samples of 2-hydroperoxyheptane and the product of the reaction with lipoxygenase-1. Total ion chromatograms are shown for methylene chloride extracts of (A) 2-hydroperoxyheptane and (B) reaction product with lipoxygenase-1 (0.1 M Tris-HCl, pH 7.0, 30 min, room temperature). Insets: mass spectra corresponding to the major components.

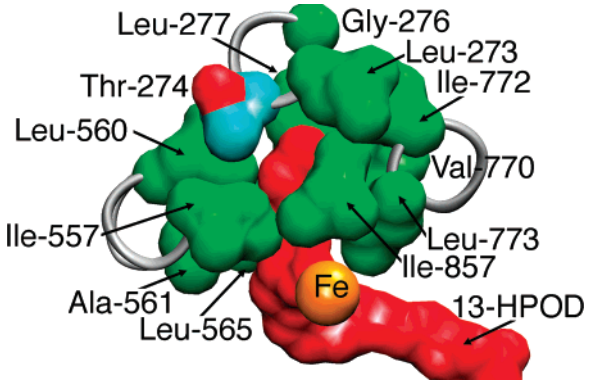


FIGURE 8: Van der Waals surfaces of the amino acid side chains (hydrophobic, green) in contact with the ω-terminal end of 13-HPOD (red) in the X-ray crystallographically determined structure of the 13-HPOD/lipoxygenase-3 complex.

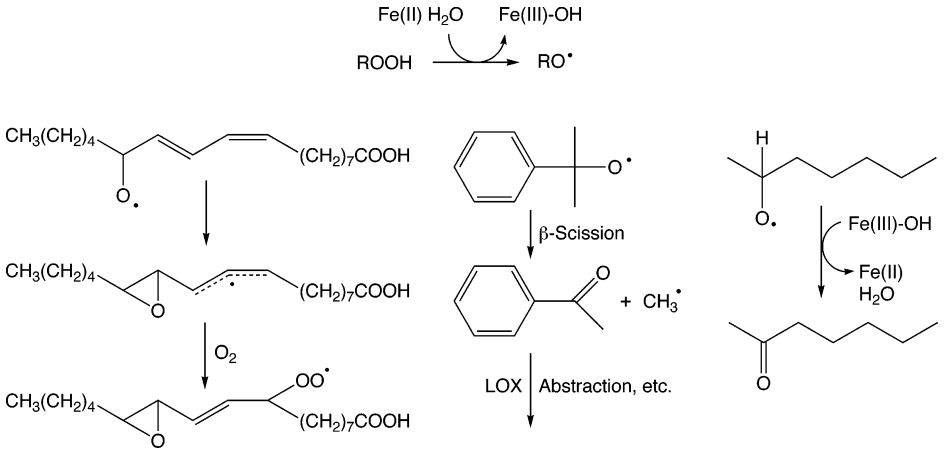
hydroperoxide moiety for accessing the active site, possibly through an interaction with the cofactor iron atom. The conformational flexibility of lipoxygenase-1 was evident in limited proteolysis and antibody binding studies (*26*). Presumably the substrate induces a change that creates a conformation with affinity for the secondary alkyl hydroperoxides. A conformational change takes place upon 13-HPOD binding, as the hydrophobic binding pocket illustrated in Figure 8 was not evident in the crystallographically determined structure of native lipoxygenase-3 (*27*).

The oxidation of the iron in lipoxygenase by the alkyl hydroperoxides was evident in EPR spectroscopic measurements. The hydroperoxides were delivered directly to the enzyme solutions as aliquots in methanol in order to avoid loss of the compounds to evaporation. The consequences for including methanol in EPR measurements of lipoxygenase-1 are illustrated in Figure 3. The 2-hydroperoxyalkanes gave rise to the same high-spin iron(III) $g = 6$ signal in the EPR spectra of lipoxygenase-1 as was obtained with 13-HPOD. The reaction between lipoxygenase-1 and the secondary alkyl hydroperoxides appears to be a two-step process. We were able to get the iron(III) form of the enzyme to build up by employing a sufficiently large excess of the hydroperoxides, so there must be a difference in the rates of the two steps. As for the kinetic data, the magnitude of the EPR signals was dependent on the length of the alkyl substituent. These observations illustrate a bipartite nature for the oxidation of lipoxygenase iron, involving affinity for the iron site and redox chemistry. The clear order of the signal intensity for the three compounds presumably reflects a higher affinity for the more hydrophobic hydroperoxides. Secondary alkyl hydroperoxides have somewhat more negative electrode potential values than unsaturated hydroperoxides, −0.24 V (vs SCE) for 2-hydroperoxypentane (*28*) compared to −0.04 V for 3-hydroperoxy-1-heptene (*29*) and +0.02 V for cumene hydroperoxide (*30*). This could also have contributed to the lower EPR signal intensities observed for 2-hydroperoxyalkanes compared to 13-HPOD.

There was no evidence for an activating effect of the 2-alkyl hydroperoxides in either instantaneous kinetic effects or preincubation experiments. The compounds clearly have access to the active site and the capacity to oxidize lipoxygenase iron. The observations provide evidence to substantiate the idea that there is more to activation than iron oxidation (*9*). The 2-alkyl hydroperoxides apparently lack a necessary feature of the structure of 13-HPOD for abolishing the lag phase. On the other hand, the secondary alkyl hydroperoxides appear to be substrates for a reaction catalyzed by the enzyme. We envision a two-step cycle in which the hydroperoxide is first reduced by lipoxygenase-iron(II) to the alkoxy radical, followed by oxidation of the radical to the corresponding carbonyl compound and regeneration of the iron(II) state. There was no evidence that the reaction was stereospecific. The secondary hydroperoxides were all prepared as racemic mixtures. The absence of any residual hydroperoxide after the reaction would indicate that both enantiomers were consumed, although they may have been consumed at different rates.

It is remarkable that three hydroperoxides interact with the enzyme in such different ways: 13-HPOD as an activator, cumene hydroperoxide as an inactivator, and 2-hydroperoxyheptane as an inhibitor and alternate substrate. The initial reaction in each case is presumably the same, oxidation of the iron and formation of an alkoxy radical. Proposed chemistry to account for the subsequent differences is offered in Scheme 3. The fate of 13-HPOD involves a cyclization reaction to produce an allylic radical (*31*). Combination of the allylic radical with molecular oxygen would be expected to result in formation of the relatively unreactive peroxyl radical. This chemistry could be fast (intramolecular) and might separate the radical in terms of physical proximity from the iron atom. The cumyloxy radical from cumene hydroperoxide could react directly with the enzyme or undergo

Scheme 3



β-scission to afford the methyl radical (*32*). In either case, free radical chemistry (abstraction) would be expected. The observation of a very conspicuous free radical signal in the EPR spectrum of cumene hydroperoxide-treated lipoxygenase-1 was consistent with this hypothesis (*9*). Also, the shape of cumene hydroperoxide may not be quite right for the hydrophobic pocket adjacent to the iron site and consequently would not be retained in close proximity to the iron. By contrast, 2-hydroperoxyheptane would be an excellent fit for the hydrophobic cavity and could remain poised to engage in a catalytic cycle with the iron. We do not see any evidence for the dissociation of the alkoxy radical from the enzyme upon treatment with the secondary alkyl hydroperoxides. That should, for example, have the same inactivating effect as for cumene hydroperoxide, which was not observed.

Secondary alkyl hydroperoxides undergo dehydration to ketones in the presence of strong acids and bases, chemistry that is presumably heterolytic in nature (*33*, *34*). Carbonyl compounds were also obtained as one member of a complex mixture of products when fatty acid hydroperoxide decomposition was induced by iron, a reaction involving a free radical mechanism (*35*, *36*). The formation of carbonyl compounds in reactions catalyzed by lipoxygenase-1 was also observed under a variety of circumstances. For example, under anaerobic conditions, Garssen et al. (*37*) obtained 13-oxo-9,11-octadecadienoic acid as one component of a mixture of products from lipoxygenase-1 acting on a combination of linoleic acid and 13-HPOD. A reaction mechanism with a peroxy radical intermediate was invoked to account for the observed products. Kuhn et al. (*38*) discovered that lipoxygenase-1 oxygenated methyl-12-keto-(9*Z*)-octadecadienoate, generating methyl-9,12-diketo-(10*E*)-octadecadienoate in the process. A mechanistic pathway through the 9-hydroperoxide and an alkoxy radical was envisioned. Gardner and Grove (*39*) found that lipoxygenase-1 converted 3*Z*-nonenal into 4-oxo-2*E*-nonenal. This was interpreted as occurring through the initial formation of a hydroperoxide product (conditions were also reported that favored the formation of this compound) and a subsequent reaction involving an alkoxy radical intermediate. Nieuwenhuizen et al. (*40*) reported that lipoxygenase-1 converted 11-hydroperoxyoctadec-10-en-9-ynoic acid into 11-oxooctadec-12-en-9-ynoic acid. A mechanism invoking intermediate hydroperoxyl and hydroxyl radicals was proposed in order to account for the enzyme inactivation observed in that system. Finally, Clapp et al. (*41*) recently showed that lipoxygenase-1 catalyzed the oxygenation of monounsaturated fatty acids. The isolated products were enones, and evidence from trapping experiments indicated that there were hydroperoxide intermediates in their formation. It was proposed that the enones formed through removal of water from the intermediate hydroperoxides. Our results provide direct experimental support for the formation of carbonyl compounds through hydroperoxide intermediates in a reaction that is catalyzed by lipoxygenase.

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