Oxygenation of Monounsaturated Fatty Acids by Soybean Lipoxygenase-1: Evidence for Transient Hydroperoxide Formation†

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ABSTRACT: Soybean lipoxygenase-1 (SBLO-1) catalyzes the oxygenation of polyunsaturated fatty acids to produce conjugated diene hydroperoxides. Previous work from our laboratories has demonstrated that SBLO-1 will also catalyze the oxygenation of monounsaturated acids (Clapp, C. H., Senchak, S. E., Stover, T. J., Potter, T. C., Findeis, P. M., and Novak, M. J. (2001) Soybean Lipoxygenase-Mediated Oxygenation of Monounsaturated Fatty Acids to Enones, *J. Am. Chem. Soc. 123*, 747−748). Interestingly, the products are α,β-unsaturated ketones rather than the expected allylic hydroperoxides. In the present work, we provide evidence that the monoolefin substrates are initially converted to allylic hydroperoxides, which are subsequently converted to the enone products. The hydroperoxide intermediates can be trapped by reduction to the corresponding allylic alcohols with glutathione peroxidase plus glutathione or with $SnCl_2$. Under some conditions, the hydroperoxide intermediates accumulate and can be detected by HPLC and peroxide assays. Kinetics measurements at low concentrations of [1-^{14}C]-9(*Z*)-octadecenoic acid indicate that oxygenation of this substrate at 25 °C, pH 9.0 occurs with $k_{cat}/K_m = 1.6\ (\pm 0.1) \times 10^2\ M^{-1}\ s^{-1}$, which is about 10^5 lower than k_{cat}/K_m for oxygenation of 9(*Z*),12(*Z*)-octadecadienoic acid (linoleic acid). Comparison of the activities of 9(*Z*)-octadecenoic acid and 12(*Z*)-octadecenoic acid implies that the two double bonds of linoleic acid contribute almost equally to the C−H bond-breaking step in the normal lipoxygenase reaction. The results are consistent with the notion that SBLO-1 functionalizes substrates by a radical mechanism.

Lipoxygenases catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids and their derivatives under physiological conditions (*1*, *2*). These enzymes take advantage of the enhanced reactivity of the 1(*Z*),4(*Z*)-diene unit found in their substrates to convert them into conjugated diene hydroperoxides. Lipoxygenases mediate the initial step in the conversion of polyunsaturated fatty acids to important signaling molecules, such as leukotrienes (*3*) and lipoxins (*4*) in animals and jasmonic acid and traumatin in plants (*5*, *6*). Some lipoxygenases also act on polyunsaturated fatty acid side chains in complex lipids and are thought to be involved in the maturation of red blood cells in mammals (*7*) and the degradation of lipid polar bodies in oil seeds (*8*). Human lipoxygenases have been implicated in asthma (*9*), inflammation (*3*, *4*), atherosclerosis (*10*), and cancer (*11*).

Most of our mechanistic insight into lipoxygenases comes from soybean lipoxygenase-1 (SBLO-1),[1] which is most active with substrates that have a 1,4-diene unit that begins on the sixth carbon from the methyl terminus (*12*, *13*). For example, SBLO-1 catalyzes the conversion of linoleic acid to 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD, Scheme 1). Highly purified SBLO-1 contains one Fe(II) ion, which is oxidized by 13-HPOD to the Fe(III) state (*14*−*17*). Crystal structures of SBLO-1 have been reported (*18*, *19*) as has the crystal structure of a closely related isozyme (SBLO-3) in complex with 13-HPOD (*20*). The crystal structure of one mammalian lipoxygenase, arachidonate 15-lipoxygenase from rabbit reticulocytes, has been determined and is similar in secondary and tertiary structure to SBLO-1 (*21*).

There is considerable evidence that catalysis by SBLO-1 is initiated by reaction of the Fe(III) form of the enzyme with the fatty acid substrate to form an intermediate, which then reacts with dioxygen (*22*−*25*). A reasonable candidate for this intermediate is a pentadienyl radical (see Scheme 2, pathway A) (*22*−*27*), which could be formed by transfer of a hydrogen atom from the substrate to a ferric hydroxide moiety (*26*, *27*). (For model studies, see refs 28 and 29.) However, direct evidence for a pentadienyl radical intermediate is lacking. Other suggestions for the nature of the intermediate include an organoiron species (Scheme 2,

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[1] Abbreviations: SBLO-1, soybean lipoxygenase-1; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; SBLO-3, soybean lipoxygenase-3; HPLC, high-performance liquid chromatography; UV, ultraviolet; FOX, ferrous-xylenol orange; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; GSH, glutathione; GPx, glutathione peroxidase; EIMS, electron impact mass spectrometry; COSY, correlation spectroscopy; 12-ODE, 12(*Z*)-octadecenoic acid; 13-HOD, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid.

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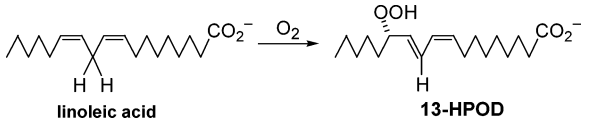
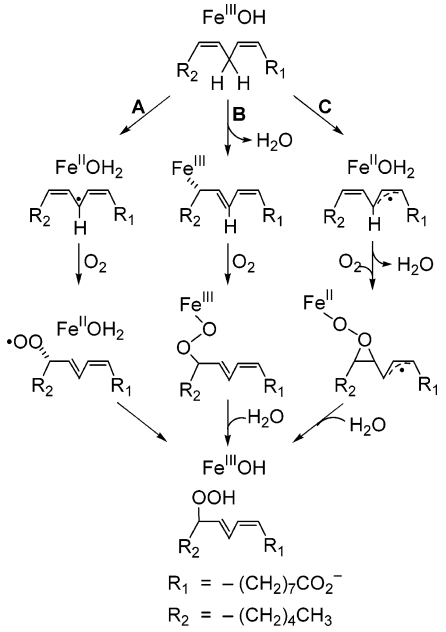
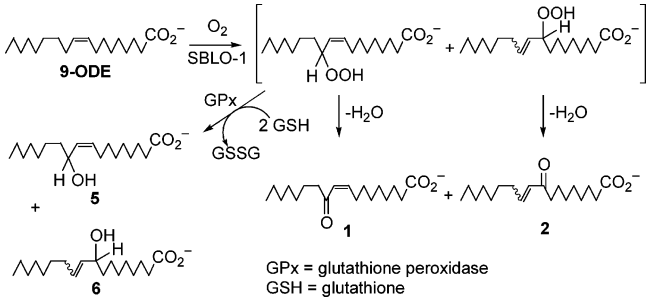
Scheme 1



Scheme 2



Scheme 3

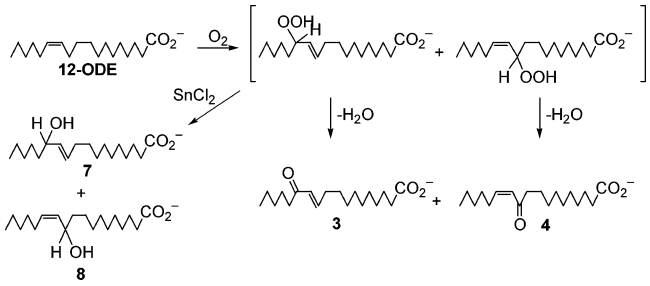


pathway B) (*30*) and a Δ^{12}-[9,10,11]-allyl radical (Scheme 2, pathway C) (*31*).

Mechanistic interest in SBLO-1 has been heightened by the discovery that the C−H bond-breaking step exhibits a very large kinetic isotope effect ($k_H/k_D = 81$) (*32*). The magnitude and very low temperature-dependence of this effect led to the proposal that hydrogen transfer occurs by tunneling (*33*), and there has been considerable interest in understanding the role of the enzyme in mediating this process (*34*−*41*). Very large isotope effects have also been observed for the oxygenation reaction catalyzed by arachidonate 15-lipoxygenase (*42*, *43*) and for an elimination reaction catalyzed by SBLO-1 (*44*).

Our laboratories have reported that SBLO-1 will slowly oxygenate monounsaturated fatty acids (*45*). Preparative application of this reaction can be facilitated by use of high pressures of O_2. Interestingly, the products are enones rather than hydroperoxides. For example, oleic acid is converted primarily to 11-oxo-9(*Z*)-octadecenoic acid (**1**, see Scheme 3). In addition, a small amount of 9-oxo-10-octadecenoic acid (**2**) is formed at normal pressures of O_2 but not at high

Scheme 4



pressures. 12(*Z*)-Octadecenoic acid is converted primarily to 13-oxo-11(*E*)-octadecenoic acid (**3**, Scheme 4) plus a smaller amount of 11-oxo-12(*Z*)-octadecenoic acid (**4**).

One possible explanation for the formation of enones is that the monoolefin substrates undergo a typical lipoxygenation reaction to form allylic hydroperoxides, which are subsequently converted to the enones by loss of water (Schemes 3 and 4). In this paper we present evidence in support of this hypothesis. We also present kinetic studies that enable us to compare the rates at which SBLO-1 functionalizes simple olefins and 1,4-dienes. The results provide insight into the general question of how lipoxygenases functionalize C−H bonds.

MATERIALS AND METHODS

Materials. SBLO-1 purified from soybeans by the procedure of Axelrod (*46*) was used for all experiments except for the trapping experiments on 12(*Z*)-octadecenoic acid, for which Type 1 lipoxygenase from Sigma was used. Oleic acid, linoleic acid, glutathione, and glutathione peroxidase (from bovine erythrocytes) were obtained from Sigma, and anhydrous $SnCl_2$ was obtained from Aldrich. 12(*Z*)-Octadecenoic acid was prepared by saponification of methyl 12(Z)-octadecenoate (Sigma). 13-HPOD was prepared by the action of SBLO-1 on linoleic acid, and 13-HOD was obtained by reduction of 13-HPOD with $NaBH_4$. [1-^{14}C]Oleic acid was obtained from Perkin-Elmer Life Sciences.

Experiments with [1-^{14}C]Oleic Acid. Aliquots of a 1.8 mM stock solution of [1-^{14}C]oleic acid (56 μCi/μmol) in ethanol were combined with unlabeled oleic acid in ethanol to prepare working solutions of the desired specific activity and a total oleic acid concentration approximately 100 times higher than the final oleic acid concentration in a particular experiment. Reactions were initiated by adding an aliquot of the appropriate [1-^{14}C]oleic acid solution to a solution of SBLO-1 and 13-HPOD in 50 mM borate, pH 9.0 that had been preincubated for 5 min at the reaction temperature. Aliquots of the reaction mixture were withdrawn at the desired times and quenched by addition of an equal volume of ice-cold acetonitrile/water/phosphoric acid (15:6:0.24) (method A) or by addition of 0.05 volume of 0.5 M citric acid followed by 0.10 volume of methanol and cooling to −80 °C (method B). Aliquots (100 μL) of the quenched solutions were analyzed by HPLC under conditions described below. If method A was used, the samples were stored at −20 °C prior to HPLC analysis; if method B was used, samples were stored at −80 °C. With both methods, the relative intensities of the HPLC peaks did not vary with storage time, which indicates that both methods completely stop the reaction. When method A was used, a portion of

the radioactivity sometimes eluted at the void volume of the HPLC column. Data from runs in which this occurred were discarded. This problem is avoided with method B.

Analytical Methods. HPLC was carried out on a Shimadzu LC10AS system with a 250 × 4.6 mm Alltech Adsorbosphere C18 5 μm column. Mobile phase A was 0.1% acetic acid in deionized water, and mobile phase B was 0.1% acetic acid in acetonitrile. Elution program 1 consisted of 3 min of isocratic elution at 60% B followed by a 14 min linear gradient from 60% to 90% B and 13 min at 90% B. Elution program 2 consisted of 3 min of isocratic elution at 50% B followed by a 14 min linear gradient from 50% to 90% B and 13 min at 90% B. The flow rate was 1.0 mL/min throughout both programs. UV-detection was carried out with a Shimadzu SPD-10 A UV−visible detector, and radioisotope detection was done with an INUS β-Ram model 2 flow detector using IN-FLOW-BD scintillation fluid at a flow rate of 2.0 mL/min.

Peroxide determinations were carried out using the ferrous−xylenol orange (FOX) method (*47*, *48*). To assay for peroxide intermediates, 100 μL aliquots of reaction mixtures were added to 900 μL of the FOX reagent (methanol/water (9:1) containing ferrous sulfate (100 μM), xylenol orange (100 μM), and H_2SO_4 (25 mM)). After 1 h at room temperature, the absorbance at 570 nm was determined and converted to peroxide concentration using a standard curve prepared with 13-HPOD.

GC/MS analyses were carried out on a Hewlett-Packard GCD instrument with a 12 m × 0.2 mm HPI capillary column (methyl silicone, 0.3 mm film thickness). NMR spectra were obtained on a Bruker 300 MHz (AXR-300) spectrometer with tetramethylsilane as the internal standard.

Derivatizations. To convert carboxylic acids to methyl esters, solutions of the acids in methanol were treated dropwise with diazomethane in ether until the yellow color persisted, and the excess diazomethane and solvents were removed under nitrogen. The diazomethane solutions were generated from 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich) using an Aldrich micro diazomethane kit. Alcohols (ca. 1 μg) were converted to their trimethylsilyl derivatives by treatment with 10 μL of BSTFA (Sigma) and 10 μL of pyridine for 1 h at room temperature. Catalytic hydrogenation was carried out by stirring unsaturated substances (ca. 1 μg) in 1.0 mL of methanol in the presence of 9.5 mg of 5% Pd on $CaSO_4$ under H_2 for 1 h at room temperature.

Oxygenation of Oleic Acid in the Presence of Glutathione/Glutathione Peroxidase. A solution of SBLO-1 (5 μM) and 13-HPOD (5 μM) in 50 mM borate, pH 9.0 (total volume = 6.1 mL) was treated with glutathione peroxidase (26 units), glutathione (final concentration = 250 μM), and oleic acid (final concentration = 75 μM). After 2.4 h at 25 °C, the reaction mixture was acidified with citric acid to pH 3 and applied to a 0.5 mL Bakerbond C18 solid-phase extraction column that had previously been washed with 10 mL of methanol and 5 mL of H_2O. After application of the sample, the column was eluted with 5 mL of H_2O followed by 6 mL of methanol. The methanol eluent was concentrated under N_2, and the residue was dissolved in methanol and derivatized with diazomethane and BSTFA as described above. GC/MS analysis showed methyl oleate, a major product, and a minor product. Mass spectra of the major and minor products are presented in panels A and B of Figure 3 in the Results.

Oxygenation of 12(Z)-Octadecenoic Acid in the Presence of $SnCl_2$. To a beaker containing a Teflon-coated stir bar and 40 mg of 12(*Z*)-octadecenoic acid was added 50 mL of chilled borate buffer (0.1 M, pH 8.7, 4 °C) with vigorous stirring. To this was added 40 mg of anhydrous $SnCl_2$. The turbid mixture was placed in a pressure chamber (Parr Instrument Co., Moline, IL) previously chilled to 4 °C in a cold room, and 250 mg of SBLO-1 (Sigma, Type 1) was added in a single portion with continued stirring. The chamber was then sealed, and pressurized to a constant 20 atm of oxygen. After stirring at 4 °C for 24 h, the oxygen supply was disconnected and the pressure chamber was slowly decompressed to avoid excess foaming of the reaction mixture. The reaction was removed from the chamber, and with stirring, the pH was adjusted to ~4 by the dropwise addition of glacial acetic acid. After passing through a plug of Celite-545, the eluent was extracted three times with 50 mL portions of diethyl ether. The organic fractions were combined, rinsed with brine, and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent in vacuo, flash chromatography utilizing a mobile phase of ethyl acetate/hexanes/formic acid (1:5:0.2) yielded 5 mg (12%) of unreacted 12(*Z*)-octadecenoic acid, 4 mg (10%) of enones **3** and **4**, and 24 mg (57%) of an inseparable mixture of **7** and **8** as a colorless solid. ^{1}H NMR ($CDCl_3$, ppm): 5.63 (1 H, dt, J = 15.4 Hz, J = 6.6 Hz), 5.45 (3 H, m), 4.43 (1 H, q, J = 6.9 Hz), 4.04 (1 H, J = 6.6 Hz), 2.35 (4 h, t, J = 7.4 Hz), 2.06 (4 H, m), 1.65−1.20 (44 H, m), 0.89 (6 H, t, J = 6.5 Hz). ^{13}C NMR ($CDCl_3$, ppm): 179.6, 179.5, 133.0, 132.6, 132.5, 132.3, 73.4, 67.9, 34.1, 32.2, 31.8, 31.6, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.1, 27.8, 25.4, 25.3, 24.7, 22.6, 14.1. A ^{1}H COSY spectrum is presented in the Supporting Information (Figure S1). EIMS of the methyl ester-trimethylsilyl derivative of **7**: *m*/*z* (rel int) 384 (M^+, 0.3), 369 (M−CH_3, 0.7), 353 (M−OCH_3, 0.3), 337 (3), 199 (100), 143 (7), 129 (33), 109 (13). EIMS of the methyl ester-trimethylsilyl derivative of **8**: *m*/*z* (rel int) 384 (M^+, 0.4), 369 (M−CH_3, 0.8), 353 (M−OCH_3, 0.7), 337 (5), 313 (100), 241 (5), 209 (16), 199 (11), 129 (21), 109 (8).

RESULTS

Trapping of Peroxide Intermediates. Our working hypothesis was that the lipoxygenase-catalyzed conversion of oleic acid to the enone products proceeded by way of allylic hydroperoxides, as shown in Scheme 3. To test this hypothesis, the reaction was carried out in the presence of glutathione (GSH) and glutathione peroxidase (GPx), which would be expected to trap the hydroperoxide intermediates by reduction to the corresponding allylic alcohols **5** and **6**. In initial small-scale experiments, [1-^{14}C]oleic acid (75 μM, 0.15 μCi/μmol) was incubated with SBLO-1 (5.0 μM) and 13-HPOD (5.0 μM) at 25 °C in the absence and presence of glutathione (250 μM) and glutathione peroxidase (1.8 units/mL). After 1 h, the reactions were analyzed by HPLC with both radiochemical and UV (234 nm) detection. The products obtained in the absence of GPx and GSH (Figure 1) have previously been identified as **1** and **2** (*45*). As expected for enones, these products are detectable by UV absorbance at 234 nm. In the presence of GPx and GSH (Figure 2), two radioactive products (**A** and **B**) were obtained with shorter retention times than **1** and **2**. These products cannot be detected at 234 nm.

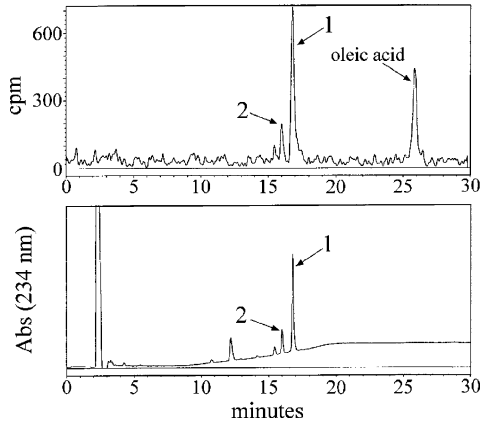


FIGURE 1: HPLC analysis of the products obtained from the oxygenation of [1-^{14}C]oleic acid by SBLO-1. The reaction conditions are described in the text. The reaction was quenched using method A, and HPLC was carried out on a 250 uL aliquot using elution program 1 with radioisotope detection (top panel) and UV detection at 234 nm (bottom panel). The small peak at 15.5 min in both the radioisotope and UV channels is due to a small amount of 11-oxo-9(*E*)-octadecenoic acid (*45*). As noted previously (*45*), this compound can arise from nonenzymatic isomerization of **1**.

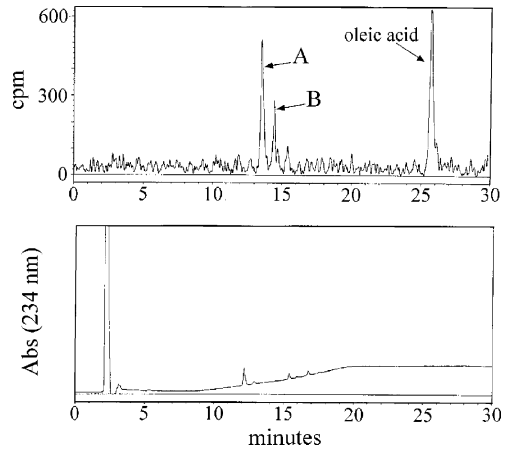


FIGURE 2: HPLC analysis of the products obtained from the oxygenation of [1-^{14}C]oleic acid by SBLO-1 in the presence of glutathione and glutathione peroxidase. The reaction conditions are given in the text, and the quench procedure and HPLC conditions are identical to those in Figure 1.

A larger-scale experiment in the presence of GPx and GSH was carried out as described in the Materials and Methods, and the products were methylated with diazomethane and treated with BSTFA to convert any hydroxyl groups to their trimethylsilyl derivatives. Two products were detectable by GC/MS analysis. The electron impact mass spectra of the major product (Figure 3A) and the minor product (Figure 3B) were consistent with the trimethylsilylated methyl esters of **5** and **6**, respectively. Both spectra show a very weak M^+ peak at *m*/*z* 384 and a prominent fragment at *m*/*z* 285 or 227 resulting from cleavage of the bond between the carbon bearing the trimethylsilyl group and the adjacent sp^3 hybridized carbon. Further evidence for the assigned structures was obtained by catalytic hydrogenation of the C_9-C_{10} double bond. Following hydrogenation, the mass spectrum of the major product gave diagnostic fragments at *m*/*z* 287 and 201 due to cleavage on both sides of the trimethylsilyl group at C11, and the spectrum of the minor product gave corresponding fragments at *m*/*z* 229 and 259 due to cleavage adjacent to the trimethylsilyl group at C9. On the basis of these results, we conclude that the products obtained in the presence of GPx and GSH are **5** (major) and **6** (minor).

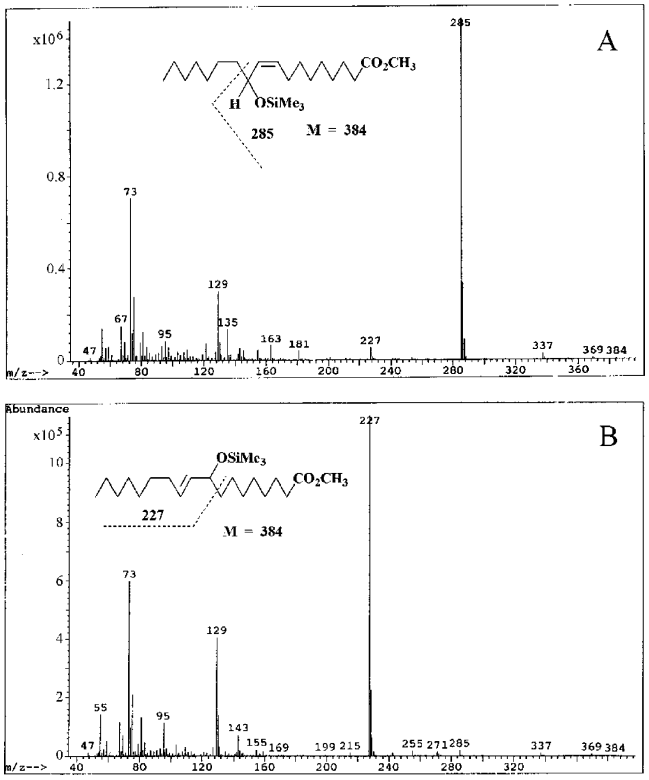


FIGURE 3: Mass spectra of the major product (panel A) and minor product (panel B) obtained from the oxygenation of oleic acid by SBLO-1 in the presence of glutathione and glutathione peroxidase.

To trap the presumed peroxide intermediates on a preparative scale in the lipoxygenase-catalyzed oxygenation of 12-(*Z*)-octadecenoic acid (12-ODE, Scheme 4), the reaction was carried out under hyperbaric conditions (20 atm of O_2) in the presence of $SnCl_2$, which reduces hydroperoxides to alcohols (*49*). Extractive workup followed by flash chromatography yielded unreacted 12-ODE (12%), enones **3** and **4** (10%), and a third fraction that gave ^{1}H and ^{13}C NMR spectra consistent with a mixture of **7** and **8** (57% yield) in approximately equal amounts. The mixture of **7** and **8** was treated with diazomethane and BSTFA and subjected to GC/MS analysis, which gave two peaks of nearly equal intensity. The mass spectrum of one component was consistent with the methyl ester of the trimethylsilyl derivative of **7**, with an M^+ peak at *m*/*z* 384 and a base peak at *m*/*z* 313 due to cleavage between C13 and C14. The mass spectrum of the other component was consistent with the methyl ester of the trimethylsilyl derivative of **8** (base peak at *m*/*z* 199; M^+ at *m*/*z* 384).

Detection of Peroxide Intermediates. Figure 4 shows the results of an experiment in which 40 μM [1-^{14}C]oleic acid (specific activity = 7.7 μCi/μmol) was incubated at 5 °C with 3.0 μM SBLO-1 in the presence of 5.0 μM 13-HPOD in 50 mM borate, pH 9.0. After 120 min, the temperature was increased to 25 °C. The reaction was monitored as a function of time by HPLC with radiochemical detection. Panel A shows the chromatogram obtained after 80 min. In addition to peaks for unreacted oleic acid and the major product, **1**, a prominent peak was observed at 17.6 min. This peak was not present at the end of the reaction (panel B). As shown in panel C, the area under the transient peak

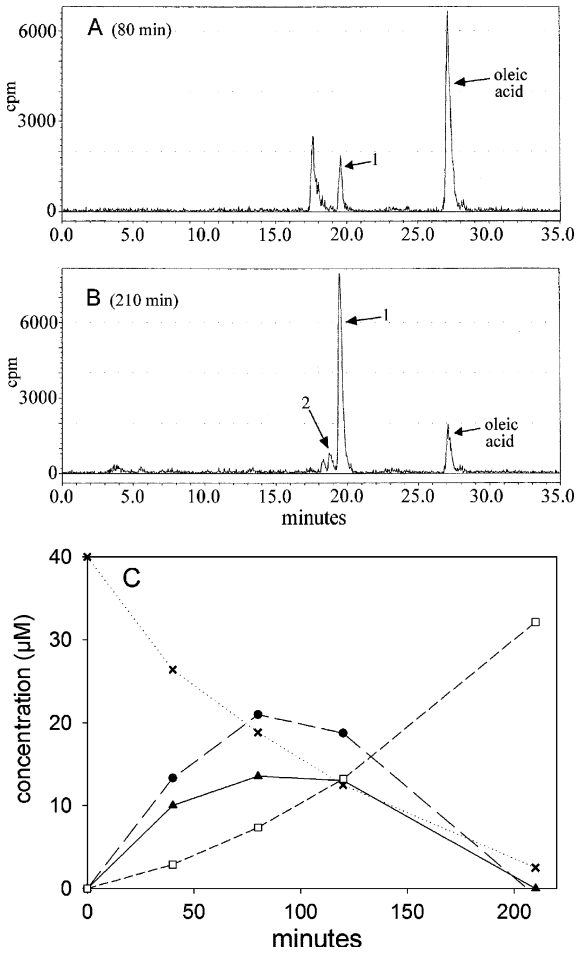


FIGURE 4: Intermediates and products in the oxygenation of oleic acid by SBLO-1 monitored by HPLC and peroxide assay. The reaction mixture contained 40 μM [1-^{14}C]oleic acid (specific activity = 7.7 μCi/μmol), 3.0 μM SBLO-1, and 5.0 μM 13-HPOD in 50 mM borate, pH 9.0. The reaction was initiated by addition of oleic acid, and the mixture was incubated for 120 min at 5 °C followed by 100 min at 25 °C. Aliquots (300 μL) were withdrawn periodically and quenched by method B, and 100-μL aliquots of the quenched solutions were analyzed by HPLC using elution program 2. (a) Chromatogram after 80 min. (b) Chromatogram after 210 min. (c) Concentrations calculated from HPLC peak areas and specific activity of [1-^{14}C]oleic acid: (×) oleic acid (retention time = 27.2 min); (▲)peak at 17.6 min; and (□) products (retention time = 18−20 min). An identical reaction was carried out with unlabeled oleic acid and monitored for peroxide (●). In the peroxide determinations, 300 μL aliquots were withdrawn and assayed (in triplicate) for peroxide using the FOX reagent, as described in Materials and Methods.

increased early in the reaction and then decreased (especially after the increase in temperature from 5 °C to 25 °C) concomitantly with an increase in the concentration of the products **1** and **2**. (The conversion of the transient peak to products is quite slow at 5 °C. The temperature was increased to 25 °C at 120 min in this experiment to speed up this conversion.) Also shown in panel C are the results from a parallel reaction with unlabeled oleic acid that was monitored by the FOX peroxide assay (*47*, *48*). The change in peroxide concentration followed the same time course as the intensity of the transient HPLC intermediate, which implies that they are due to the same species. In this and other experiments, the calculated concentration of the species giving rise to the transient HPLC peak was consistently lower than the peroxide concentration calculated from the FOX assay. This

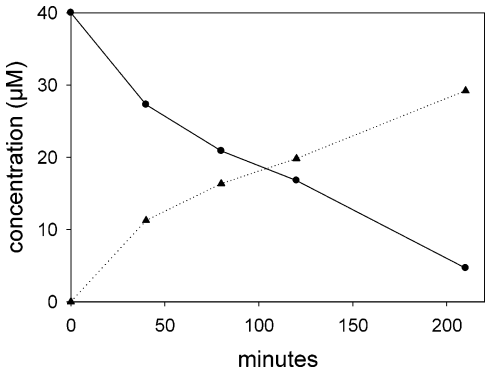


FIGURE 5: Time course of oleic acid consumption (●) and product formation (▲) when 40 μM [1-^{14}C]oleic acid was incubated with 3.0 μM SBLO-1 and 3.0 μM 13-HPOD. The reaction conditions are identical to those in Figure 4 except that the initial 13-HPOD concentration was 3.0 μM rather than 5.0 μM.

discrepancy may be due to loss of some of the peroxide prior to HPLC analysis or perhaps on the column. (Our initial attempts to isolate this intermediate indicate that it is slightly unstable under conditions of extraction and chromatography.) This loss is less likely in the peroxide assay, because aliquots of the reaction mixture were added directly to the FOX assay mix. In light of the trapping experiments, we conclude that the transient HPLC signal and peroxide are due to the hydroperoxide intermediates shown in Scheme 3.

The accumulation of peroxide intermediates is only seen under certain conditions. Running the reaction at 5 °C rather than 25 °C favors accumulation of the intermediates. In addition, a small excess of 13-HPOD relative to enzyme is essential for the accumulation of the intermediates. In the experiment described in Figure 4, 13-HPOD (5.0 μM) was present in excess over SBLO-1 (3.0 μM), so that about 2 μM 13-HPOD was expected to be present after all of the SBLO-1 had been oxidized from the ferrous to the ferric form. The presence of excess 13-HPOD was confirmed by the FOX assay immediately after the addition of enzyme, and the FOX assay data in Figure 4 were corrected for this contribution. (This correction assumes that the excess 13-HPOD remains intact over the course of the reaction. It is not certain whether this assumption is correct. If it is not, then the concentration of the peroxide intermediate at later times may be underestimated by as much as 2 μM. This small underestimation would not affect our interpretation of this figure.) When the experiment depicted in Figure 4 was repeated at an initial 13-HPOD concentration of 3.0 μM, rather than 5.0 μM, neither the transient HPLC peak nor the transient peroxide was detected. In this experiment (Figure 5), the loss of oleic acid occurred at about the same rate as in Figure 4C, and the products were formed without the lag that is observed in Figure 4C. When the experiment in Figure 5 was repeated in the presence of 2.0 μM 13-HOD (data not shown), neither the transient HPLC peak nor the transient peroxide were detected, and the rates of oleic acid consumption and product formation were virtually identical to what was observed in Figure 5. This result establishes that the peroxide moiety of 13-HPOD, not simply the carbon skeleton, must be present in excess in order to observe the accumulation of the intermediates depicted in Figure 4C.

Kinetics. Experiments at different initial concentrations of [1-^{14}C]oleic acid indicated that the rate of the reaction is optimal at about 50 μM. At higher substrate concentrations,

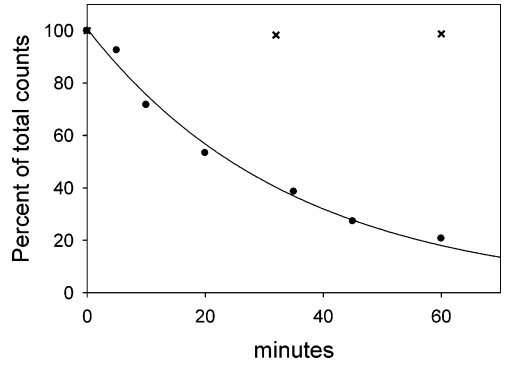


FIGURE 6: (●) Time course of oleic acid consumption (initial conc = 2.0 μM) in the presence of SBLO-1 (3.0 μM) and 13-HPOD (3.0 μM). Aliquots were quenched by method B and analyzed by HPLC using elution program 2. For each aliquot, the radioactivity in the oleic acid peak, expressed as a percentage of the total radioactivity in the chromatogram, is plotted versus time. The data were fit to the equation for first-order decay using Sigma Plot (Jandel Scientific) to give the solid line and $k_{obs} = (4.8 \pm 0.3) \times 10^{-4}$ s^{-1}. A control experiment demonstrated that SBLO-1 loses less than 5% of its activity in 1 h under the conditions of this experiment. (×) Results of a control experiment from which SBLO-1 was omitted.

the rate is reduced. This reduction in rate may be due to binding of oleic acid at an allosteric inhibitory site that was proposed by Holman and co-workers (*42*, *50*) in order to explain the effects of oleic acid on the kinetic isotope effect observed for SBLO-1. The existence of this second site is further supported by the observation that inhibition of SBLO-1 by oleyl sulfate is mixed with respect to substrate (*50*).

In order to avoid the inhibition that occurs at high oleic acid concentration, we decided to quantify the rate of oleic acid oxygenation by measuring k_{cat}/K_m at very low oleic acid concentration (*51*, *52*). Figure 6 shows the decrease in [1-^{14}C]-oleic acid over time in an experiment in which 2.0 μM [1-^{14}C]oleic acid (specific activity = 56 μCi/μmol) was incubated at 25 °C with 3.0 μM SBLO-1 and 3.0 μM 13-HPOD in 50 mM borate, pH 9.0. The initial concentration of oleic acid in this experiment is expected to be well below the K_m for oleic acid, because the reported K_i for oleic acid as a competitive inhibitor is 22 μM (*53*). The decrease in oleic acid concentration in Figure 6 follows first-order kinetics, as expected if [S] $\ll K_m$. Dividing k_{obs} by the enzyme concentration gives $k_{cat}/K_m = (1.6 \pm 0.1) \times 10^2$ M^{-1} s^{-1}. Decreasing the temperature to 5 °C lowered k_{cat}/K_m to $(1.01 \pm 0.03) \times 10^2$ M^{-1} s^{-1}. No transient intermediates were detected at either 25 °C or 5 °C.

When the experiment in Figure 6 was repeated at 25 °C using 5.0 μM 13-HPOD, k_{cat}/K_m increased slightly to $(1.8 \pm 0.1) \times 10^2$ M^{-1} s^{-1}(data not shown). Under these conditions, the transient HPLC peak observed in Figure 4a was detectable at the 5 and 10 min time points. When the reaction was carried out in the absence of 13-HPOD, no consumption of oleic acid was observed over 30 min, consistent with previous observations (*45*).

DISCUSSION

The results presented here demonstrate that the reaction catalyzed by SBLO-1 on monounsaturated fatty acids proceeds in two stages: a lipoxygenation reaction to give a mixture of allylic hydroperoxides followed by conversion of the hydroperoxides to enones. The transient hydroperoxides can be trapped by reduction to the corresponding alcohols. In the case of oleic acid, the trapping reaction was carried out at atmospheric pressure with glutathione plus glutathione reductase as the reducing system. With 12(*Z*)-octadecenoic acid, the trapping reaction was carried out under hyperbaric conditions with stannous chloride as the reductant. Stannous chloride has been used previously by Brash and co-workers to trap the transiently formed peroxide in the reaction catalyzed by the peroxidase−lipoxygenase fusion protein from coral (*49*).

Table 1: Rate Constants for the Oxygenation of Linoleic Acid and Its Monounsaturated Analogues by SBLO-1 at 25 °C, pH 9.0

substrate	k_{cat}/K_m (M^{-1} s^{-1})
oleic acid	$(1.6 \pm 0.1) \times 10^2$
12(*Z*)-octadecenoic acid	$(7.5 \pm 1.4) \times 10^2$
linoleic acid	$(1.7 \pm 0.2) \times 10^7$

Under some conditions, the hydroperoxide intermediates accumulate and can be detected by HPLC and a peroxide assay. Accumulation of the intermediates is favored by lower temperature and is only observed if 13-HPOD is present in excess over SBLO-1 at the beginning of the reaction. In the trapping experiments, 13-HPOD was stoichiometric with the enzyme, and the expected allylic alcohols were still formed. This result implies that the hydroperoxide intermediates are still formed when 13-HPOD is not present in excess, but these intermediates do not accumulate under these conditions. A possible explanation for the 13-HPOD dependence is that the conversion of the hydroperoxides to the enones is catalyzed by the ferrous form of SBLO-1. During turnover of linoleic acid by ferric lipoxygenase, radicals occasionally dissociate from the enzyme, leaving the enzyme in the ferrous form (*22*, *54*). If a similar process occurred during turnover of oleic acid, small amounts of ferrous lipoxygenase would be present, unless 13-HPOD was present in excess. These ideas are under investigation.

Formation of carbonyl products rather than (or in addition to) the expected hydroperoxides has previously been reported for SBLO-1-catalyzed reactions on $\beta-\gamma$-unsaturated carbonyl compounds (*55*, *56*) and acetylenic analogues of 1,4-dienes (*57*, *58*). In each of these cases, it was proposed that hydroperoxides are formed initially and subsequently converted to the carbonyl compounds. In their studies on the oxygenation of 3(*Z*)-nonenal by SBLO-1, Gardner and Grove (*56*) reported an effect of 13-HPOD that is similar to what we have observed with oleic acid. In the absence of 13-HPOD, 3(*Z*)-nonenal was converted primarily to 4-oxo-2(*E*)-octenal, and a smaller amount of 4-hydroperoxy-2(*E*)-nonenal was also formed. In the presence of excess 13-HPOD, 4-hydroperoxy-2(*E*)-nonenal was the major product.

Because our results establish that the initial processing of monounsaturated fatty acids by SBLO-1 occurs by a normal lipoxygenase reaction, it is of interest to compare the rates of these reactions with that for oxygenation of linoleic acid by SBLO-1. Table 1 presents k_{cat}/K_m values for the lipoxygenase catalyzed oxygenations of oleic acid, 12(*Z*)-octadecenoic acid, and linoleic acid. The value for 12(*Z*)-octadecenoic acid was calculated from the value for oleic acid using the results of previously reported competition experiments (*45*), which demonstrated that k_{cat}/K_m for

oxygenation of 12(*Z*)-octadecenoic acid is greater than that for oleic acid by a factor of 4.7 ± 0.8. The value for linoleic acid was calculated from values of k_{cat} and K_m obtained in our laboratory using conventional Michaelis−Menten kinetics. Previous work has shown that the k_{cat}/K_m value for arachidonic acid obtained by measurement at very low substrate concentration agrees well with the value calculated from conventional kinetics (*52*).

The results in Table 1 indicate that SBLO-1 functionalizes the monounsaturated substrates 4 to 5 orders of magnitude more slowly than it functionalizes linoleic acid. The relatively small difference in reactivity between the two monounsaturated fatty acids implies that both double bonds in linoleic acid contribute about equally to the rate-limiting C−H abstraction step with this substrate. This is consistent with a radical mechanism (Scheme 2, pathway A), because the absence of either double bond forces the reaction to proceed through an allylic radical rather than the more stable pentadienyl radical. To a first approximation, it should not matter which double bond is absent.

Katiguchi et al. (*59*) have recently reported that density functional theory calculations indicate that the energy difference between oleic acid and its allylic radical is 12 ± 2 kcal/mol greater than the difference between linoleic acid and the corresponding pentadienyl radical. This difference is more than enough to account for the 10^4-10^5 rate difference in the lipoxygenase-catalyzed oxygenations of linoleic acid and the corresponding monounsaturated fatty acids. Katiguchi et al. (*59*) also report kinetics results that show that the activation energy for abstraction of the allylic hydrogens in oleic acid by the cumylperoxy radical is 3.0 kcal/mol higher than that for abstraction of the bisallylic hydrogens of linoleic acid by the cumylperoxy radical. This difference corresponds to a rate difference of 160 at 25 °C, which is considerably less than the relative k_{cat}/K_m values with SBLO-1. It is possible that the abstraction catalyzed by SBLO-1 is more sensitive to the stability of the developing unsaturated radical than is the abstraction by the cumylperoxy radical or that some of the difference in k_{cat}/K_m that we observe is due to binding interactions with the enzyme. The abstraction of deuterium from 11,11-dideuteriolinoleic acid by the cumylperoxy radical was found (*59*) to exhibit an isotope effect of 6, which is much smaller than the effect of 81 observed with SBLO-1. This observation suggests that the abstraction by the cumylperoxy radical occurs by a pathway in which tunneling is much less important than in the case of SBLO-1.

The formation of mixtures of products from the monoolefin substrates is also consistent with a radical mechanism. Hydrogen abstraction from oleic acid would generate an allylic radical, which could react with O_2 at either C_9 or C_{11}, and hydrogen abstraction from 12(*Z*)-octadecenoic acid would produce an allylic radical that could react at either C_{11} or C_{13}. The products we observe are consistent with these expectations. The partitioning of the radical derived from oleic acid appears to be sensitive to oxygen concentration, because oxygenation at C_9 is observed at atmospheric pressure but not under hyperbaric conditions. It is noteworthy that all of the observed products result from initial hydrogen abstraction from C_{11}. This finding indicates that the positional specificity for hydrogen abstraction by SBLO-1 is retained even when one of the double bonds is absent.

The results are more difficult to reconcile with the mechanisms depicted in pathways B and C in Scheme 2. In each of these mechanisms, one of the two double bonds plays a more critical role than the other in C−H bond cleavage. According to the mechanism in pathway B, the presence of the $C_{12}-C_{13}$ double bond is essential for the formation of the organoiron intermediate; the C_9-C_{10} double bond may be important but is not essential. If SBLO-1 functioned by this mechanism, one would predict that it would oxygenate 12-ocatadecenoate acid much more readily than it would oxygenate oleic acid. Our results indicate that k_{cat}/K_m of 12(*Z*)-octadecenoic acid is only slightly greater than that of oleic acid, which implies that the C_9-C_{10} double bond contributes nearly as much as the $C_{12}-C_{13}$ double bond to the C−H bond cleavage. In the mechanism in pathway C, the C_9-C_{10} double bond is crucial to the stability of the Δ^{12}-[9, 10, 11]-allyl radical; the $C_{12}-C_{13}$ double bond is not. If SBLO-1 operated by this mechanism, one would expect that it could functionalize oleic acid much more readily than 12(*Z*)-octadecenoic acid. This is contrary to the results we observe. A recent study of the lipoxygenase reaction using density functional theory led to the conclusion that the reaction involving a pentadienyl radical is the most favorable of the three mechanisms depicted in Scheme 2 (*60*). Our results are consistent with this conclusion.

The regiochemical distribution of allylic alcohols obtained by oxygenation of 12(*Z*)-octadecenoic acid in the presence of $SnCl_2$ in the hyperbaric protocol deserves comment. The allylic alcohols (**7** and **8**) were isolated with an approximate 1:1 isomeric ratio, compared with the 5:1 ratio of isomeric enones produced in the absence of the reducing agent (*45*). It is known that allylic hydroperoxides can rearrange (*61*), with the most likely mechanism being a concerted suprafacial 3,2-pathway (*62*, *63*), which is different from the β-scission−recombination mechanism associated with the rearrangement of dienyl hydroperoxides such as 13-HPOD (*64*, *65*). It was tempting to speculate that this type of rearrangement would account for the ratio of isomeric allylic alcohols. However, ^{1}H NMR examination of the enone side product isolated in conjunction with the allylic alcohols revealed that the mixture was primarily 13-oxo-11(*E*)-octadecenoic acid. Its 5 fold excess in the mixture is basically identical to the enone mixture observed in our prior work (*45*). This demonstrates that the transient hydroperoxide is not rearranging prior to interaction with, and reduction by, the stannous chloride. If it were happening, the isomeric ratio of the enone products should be consistent with that of the allylic alcohols because they both arise from a common intermediate.

At this stage, we hypothesize that the stannous chloride is possibly inserting into the hydroperoxide after initial complexation, followed by transfer of the hydroxy ligand to the π-system and cleavage of the C−O bond. It must also be noted that we were not able to find evidence in the literature to either support or refute Sn(II)-mediated insertion into hydroperoxides or Sn(II)-mediated rearrangements of allylic hydroperoxides. Consequently, the nature of this apparent hydroperoxide rearrangement is currently under investigation in our laboratories.

SUPPORTING INFORMATION AVAILABLE

^{1}H−COSY spectrum of the mixture of **7** and **8** obtained by oxygenation of 12(*Z*)-octadecenoic acid by SBLO-1 in

the presence of $SnCl_2$. This material is available free of charge on the Internet at http://pubs.acs.org.

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