Irreversible Inactivation of Soybean Lipoxygenase-1 by Hydrophobic Thiols[†]

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ABSTRACT: Soybean lipoxygenase-1 is inactivated by micromolar concentrations of the following hydrophobic thiols: 1-octanethiol, 12(*S*)-mercapto-9(*Z*)-octadecenoic acid (S-12-HSODE), 12(*R*)-mercapto-9(*Z*)-octadecenoic acid (R-12-HSODE), and 12-mercaptooctadecanoic acid (12-HSODA). In each case, inactivation is time-dependent and not reversed by dilution or dialysis. Inactivation requires 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD), which suggests that it is specific for the ferric form of the enzyme. Lipoxygenase catalyzes an oxygenation reaction on each of the aforementioned thiols, as judged by the consumption of O_2. These reactions also require 13-HPOD. 1-Octanethiol is converted to 1-octanesulfonic acid, which was identified by GC/MS of its methyl ester. The rates of oxygen uptake for R- and S-12-HODE are about 5- and 2.5-fold higher than the rate with 1-octanethiol. The stoichiometries of inactivation imply that inactivation occurs on approximately 1 in 18 turnovers for 12-HSODA, 1 in 48 turnovers for 1-octanethiol, 1 in 63 turnovers for S-12-HSODE, and 1 in 240 turnovers for R-12-HSODE. These data imply that close resemblance to lipoxygenase substrates is not a crucial requirement for either oxidation or inactivation. Under the conditions of our experiments, inactivation was not observed with several more polar thiols: mercaptoethanol, dithiothreitol, L-cysteine, glutathione, *N*-acetylcysteamine, and captopril. The results imply that hydrophobic thiols irreversibly inactivate soybean lipoxygenase by a mechanism that involves oxidation at sulfur.

Lipoxygenases are non-heme iron proteins that catalyze the oxygenation of 1,4-dienes to produce conjugated diene hydroperoxides (*1*, *2*). Plant lipoxygenases are involved in the synthesis of plant growth regulators and have been implicated in development, wound healing, defense against pathogens, and other functions (*3*−*6*). Mammalian lipoxygenases (*7*) are involved in the conversion of arachidonic acid into mediators of inflammation and anaphylaxis (*8*), and lipoxygenases have also been implicated in red cell maturation (*9*), the development of atherosclerotic lesions (*10*), and tumor metastasis (*11*). Because of the widespread occurrence and physiological importance of lipoxygenases, the discovery of new inhibitors of these enzymes is of considerable interest.

Much of our fundamental chemical insight into lipoxygenases has come from studies on soybean lipoxygenase-1, which acts on polyunsaturated fatty acids and their derivatives in which a 1,4-diene unit begins on the sixth carbon from the methyl terminus (*3*, *4*, *12*). For example, 9(*Z*),12(*Z*)-octadecadienoic acid (linoleic acid) is converted to 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD).[1] In freshly purified lipoxygenase-1, most of the iron is in the Fe(II) state, which can be oxidized to the catalytically active Fe(III) state by 13-HPOD (*12*−*15*). The crystal structure of the Fe(II) form of lipoxygenase-1 has been determined (*16*, *17*), and the structure of a complex of the Fe(III) form of soybean lipoxygenase-3 with 13-HPOD has recently been reported (*18*).

Previous studies (*19*, *20*) from this laboratory have demonstrated that the ferric form of soybean lipoxygenase-1 is irreversibly inactivated by 12-iodo-9(*Z*)-octadecenoic acid (12-IODE) but not by the corresponding bromide (12-BrODE). Recent work using 11,11-dideuterio-12-IODE has demonstrated that inactivation by 12-IODE is initiated by cleavage of the C_{11}−H bond (*21*). An earlier hypothesis was that inactivation by 12-IODE might be triggered by loss of one electron from the iodine atom to the ferric ion, a process that should be more difficult with a bromine atom. This hypothesis proved to be incorrect for 12-IODE, but it led us to synthesize the *S* and *R* enantiomers of 12-mercapto-9(*Z*)-octadecenoic acid (12-HSODE, see Scheme 1), since loss of an electron from the thiol groups in these compounds would be expected to be facile. In this paper, we report that these substances and other hydrophobic thiols irreversibly

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[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 12-IODE, 12-iodo-9(*Z*)-octadecenoic acid; 12-BrODE, 12-bromo-9(*Z*)-octadecenoic acid; S-12-HSODE, 12(*S*)-mercapto-9(*Z*)-octadecenoic acid; R-12-HSODE, 12(*R*)-mercapto-9(*Z*)-octadecenoic acid; 12-HSODA, 12-mercaptooctadecanoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; DMF, *N*,*N*-dimethylformamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; CI, chemical ionization; EI, electron impact; TLC, thin-layer chromatography.

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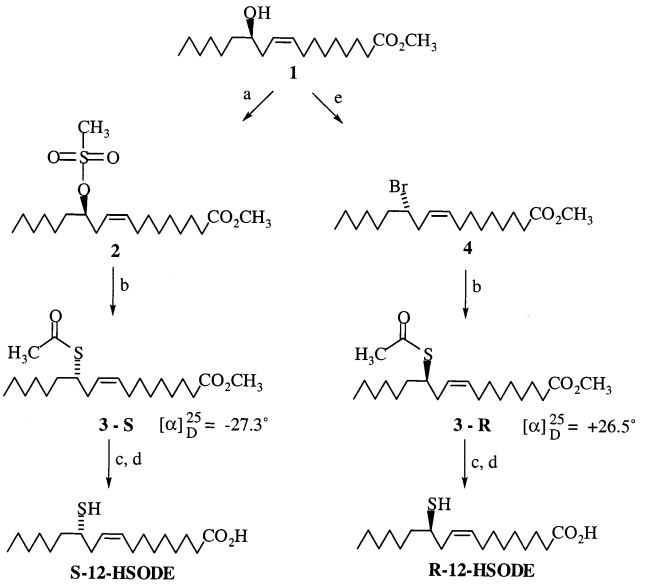
Scheme 1[a]



[a] (a) CH_3SO_2Cl, Et_3N. (b) CH_3COSK, DMF. (c) 6 M HCl, CH_3CO_2H. (d) NH_2OH. (e) CBr_4, Ph_3P, ether.

inactivate soybean lipoxygenase and also undergo lipoxygenase-catalyzed oxidation at sulfur.

MATERIALS AND METHODS

General Synthetic Methods. The syntheses of S- and R-12-HSODE are outlined in Scheme 1. Methyl (*R*)-ricinoleate (**1**) was prepared by methanolysis of castor oil according to the procedure of Swern and Jordan (*22*). To prepare S-12-HSODE, methyl (*R*)-ricinoleate was first converted to its methanesulfonate, **2**, by the procedure of Crossland and Servis (*23*). The methanesulfonate group was then displaced by thioacetate as described by Gundstone et al. (*24*); this reaction is expected to proceed with inversion of configuration to give **3-S**. Acid hydrolysis of the methyl ester in **3-S** followed by cleavage of the thioester with hydroxylamine afforded S-12-HSODE. To prepare R-12-HSODE, the hydroxyl group of methyl (*R*)-ricinoleate was displaced by bromine in a reaction that should proceed with inversion. Subsequent displacement of bromide with potassium thioacetate should invert the configuration again to produce **3-R**, and optical rotation data (see Scheme 1) confirmed that this substance had the opposite configuration to that of **3-S**. Hydrolysis of the ester and cleavage of the thioester gave R-12-HSODE. Derivatization of each enantiomer with Mosher's reagent (*25*, *26*) followed by HPLC demonstrated that each enantiomer of 12-HSODE had an enantiomeric purity of >98%.

Experimental procedures for the new reactions are presented below. In these procedures, organic extracts were dried with $MgSO_4$ prior to concentration. Flash chromatography (*27*) was carried out using silica gel 60 (230−400 mesh, Aldrich).

S-12-HSODE. A solution of 150 mg (0.41 mmol) of **3-S** in 1.2 mL of 2.0 M HCl and 4.8 mL of glacial acetic acid was prepared in a Pierce hydrolysis tube and degassed by three freeze/thaw cycles under vacuum. The tube was then closed and heated at 75 °C for 1 h. After cooling, the reaction mixture was partitioned between 10 mL of ethyl acetate and 10 mL of water, and the organic layer was concentrated to an oil. The ^{1}H NMR of this material was identical to that of **3-S** except that it lacked the singlet at 3.66 ppm due to the methyl group of the methyl ester. The oil was dissolved in a solution of hydroxylamine hydrochloride (590 mg) in ethanol (8.5 mL), H_2O (2.6 mL), and triethylamine (0.60 mL). After 30 min, 15 mL of H_2O was added, and the resulting milky suspension was acidified with 6 M HCl and then extracted with three 15-mL portions of ethyl acetate. The combined organic extracts were concentrated, and the residue was purified by flash chromatography (hexanes/acetic acid, 98:2) to give 66 mg (52%) of S-12-HSODE. ^{1}H NMR ($CDCl_3$): δ 5.44 (m, 2H), 2.85 (m, 2H), 2.35 (m, 3H), 2.04 (m, 2H), 1.2−1.8 (m, 20 H), 0.88 (t, 3H).

R-12-HSODE. A solution of methyl (*R*)-ricinoleate (1.32 g, 4.23 mmol), triphenylphosphine (1.85 g, 7.05 mmol), and carbon tetrabromide (2.70 g, 8.15 mmol) in 10 mL of anhydrous ether was stirred for 1 h at room temperature. A solid precipitate was removed by filtration, and the filtrate was concentrated to an oil, which was purified by flash chromatography (hexanes/ethyl acetate,15:1) to give **4** (1.05 g). A solution of **4** (1.05 g, 2.8 mmol) and potassium thioacetate (616 mg, 5.41 mmol) in 6 mL of DMF was stirred at room temperature for 24 h. Extraction and purification by flash chromatography (hexanes/ethyl acetate, 29:1) yielded 1.0 g of **3-R**. Hydrolysis of the ester and cleavage of the thioester were carried out as described above for the *S* isomer to yield R-12-HSODE.

Stereochemical Analysis of S- and R-12-HSODE. S- or R-12-HSODE (5 μmol) in 275 μL of ethanol was converted to its methyl ester by treatment with ethereal diazomethane. The solvents were evaporated under a stream of nitrogen, and the residue was treated with a 0.1-mL aliquot of a solution that contained (*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (38 mg), triethylamine (15 mg), and 4-(dimethylamino)pyridine (5 mg) in 1.0 mL of CH_2Cl_2. This mixture was left overnight at room temperature and then treated with one drop of dimethylaminopropylamine to destroy the unreacted acid chloride. The resulting thioester derivatives were detectable by UV absorbance, and each derivative was purified by chromatography on a 7 × 0.6 cm column of silica gel with hexanes/ethyl acetate (4:1) as elution solvent. The purified derivatives were dissolved in 200 μL of isooctane, and these solutions were further diluted 1:10 with isooctane for HPLC analysis.

Normal-phase HPLC analysis of the diastereomeric thioester derivatives was performed on a 250 × 4.6 mm Alltech Adsorbosphere silica gel (5 μm) column with isocratic elution using isooctane/ethyl acetate (83:1) as mobile phase. Detection was by UV-absorbance at 254 nm. The derivative of S-12-HSODE gave a major peak eluting at 11.7 min and a barely detectable peak (<2% of major peak) at 10.6 min. The derivative from R-12-HSODE gave a major peak at 10.6 min and a minor peak (<2%) at 11.7 min. These results imply that each enantiomer had a stereochemical purity of >98%.

Other Thiols. Racemic 12-mercaptooctadecanoic acid (12-HSODA) was synthesized from racemic methyl 12-hydroxystearate (Sigma) by the same sequence of reactions used to prepare R-12-HSODE from methyl (*R*)-ricinoleate. 1-Octanethiol, *N*-acetylcysteamine, and 2-mercaptoethanol were from Aldrich, and L-cysteine, dithiothreitol, glutathione, and captopril were from Sigma.

Enzymes and Assays. Soybean lipoxygenase-1 was purified by the method of Axelrod (*28*), and lipoxygenase concentrations were determined spectrophotometrically using $A^{0.1\%}_{280\ nm} = 1.6$ (*29*). Spectrophotometric assays of lipoxygenase activity were carried out at 25 °C in reaction mixtures containing 72 μM linoleic acid and 2 μM 13-HPOD in 50 mM borate buffer, pH 9.0. The initial rate of 13-HPOD formation was measured at 234 nm. Bovine liver catalase was obtained from Sigma Chemical Co. and had a specific activity of 1350 units/mg when assayed by the procedure provided by the supplier. Oxygen consumption was measured polarographically with a Clarke-type electrode using a Hansatech D. W. Oxygen Electrode Unit.

Assay of Thiol Consumption. Lipoxygenase (2 μM), 13-HPOD (20 μM), and S-12-HSODE (50 μM) were incubated at 25 °C in 50 mM borate buffer, pH 9.0 (total volume = 500 μL). The reaction was initiated by addition of S-12-HSODE and terminated after 2−15 min by addition of an ice-cold mixture of 500 μL of ethanol plus 40 μL of 10 mM DTNB in 0.1 M potassium phosphate buffer, pH 8.0 (*30*). The mixture was centrifuged for 3 min at 3 °C, and the absorbance was read at 412 nm relative to a blank from which both enzyme and S-12-HSODE were excluded. Thiol concentration was calculated using $\epsilon_{412} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ (*30*); experiments with L-cysteine indicated that this extinction coefficient was not significantly affected by the ethanol present. The contribution of protein thiol groups was determined by a control from which S-12-HSODE was omitted.

Oxygenation of 1-Octanethiol by Lipoxygenase. A solution of 4 μM lipoxygenase and 5 μM 13-HPOD in 50 mM borate, pH 9.0 (total volume = 4.9 mL), at 3 °C was treated with 100 μL of 2.5 mM 1-octanethiol in ethanol. After 2 min, the reaction was quenched with a few drops of 6 M HCl, and the solution was immediately loaded onto a 0.5-mL Bakerbond C18 solid-phase extraction column that had been previously washed with 10 mL of methanol and 5 mL of deionized water. After application of the sample, the column was washed with 5 mL of water, and the products were eluted with 6 mL of methanol. The methanol eluate was concentrated to dryness, and the residue was dissolved in methanol (25 μL) and treated with excess ethereal diazomethane. The resulting solution was treated with a few drops of acetic acid to destroy the excess diazomethane, concentrated, and redissolved in methanol (30 μL) for GC and GC/MS analysis. Control samples were prepared identically, starting with reaction mixtures from which either lipoxygenase or 1-octanethiol were omitted. To obtain a standard solution of methyl 1-octanesulfonate and to validate our extraction/derivatization procedure, a 0.50-mL aliquot of a 50 mM solution of sodium 1-octanesulfonate (Aldrich) in 50 mM borate, pH 9.0, was acidified, subjected to solid-phase extraction, and treated with diazomethane, exactly as described above for the enzymatic reaction.

GC and GC/MS. Gas chromatography was carried out on a Hewlett-Packard 5890 instrument with a flame ionization detector and a 12 m × 0.2 mm HP1 capillary column (methyl silicone, 0.3 mm film thickness) at a helium flow rate of about 1.5 mL/min. The temperature program was as follows: initial temperature = 50 °C for 2 min followed by an increase of 20 °C/min up to 250 °C and elution at 250 °C for 2 min. GC/MS analyses were carried out under the same chromatography conditions using either a Hewlett-Packard GCD instrument (for EI spectra) or a Hewlett-Packard 5890 GC interfaced with an Extrel ELQ 400 mass spectrometer (for CI spectra). CI spectra were obtained with isobutane as reagent gas.

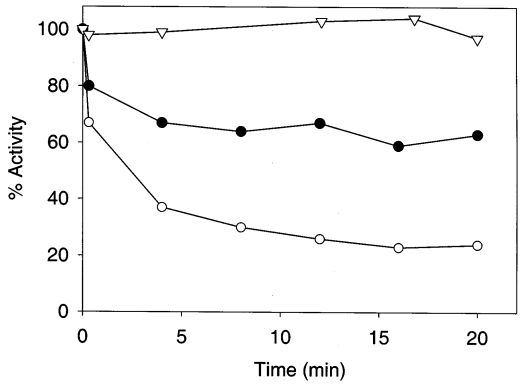


FIGURE 1: Inactivation of lipoxygenase by S-12-HSODE. Lipoxygenase (0.20 μM) was incubated at 25 °C with (●) 5.0 μM S-12-HSODE, (○) 10 μM S-12-HSODE, or (▽) no S-12-HSODE in 50 mM borate, pH 9.0, containing 2.0 μM 13-HPOD and 0.075% ethanol. Lipoxygenase was added at time zero, and 15 μL aliquots were withdrawn periodically and assayed spectrophotometrically in a total volume of 1.0 mL as described under Materials and Methods.

RESULTS

Inactivation by S-12-HSODE. When 10 μM S-12-HSODE was incubated with 0.2 μM lipoxygenase in the presence of 2 μM 13-HPOD, a rapid drop in activity occurred (Figure 1). When the activity stabilized after 16−20 min, about 77% of the initial activity had been lost. When 5 μM S-12-HSODE was used, 39% of the activity was lost after 16−20 min. No loss of activity occurred in the absence of S-12-HSODE. To obtain each point in Figure 1, an aliquot of the incubation mixture was diluted 67-fold into an assay mixture that contained 72 μM linoleic acid and 2 μM 13-HPOD. Thus, the S-12-HSODE concentration in the final assays was 0.15 or 0.075 μM. Control experiments demonstrated that 0.15 μM S-12-HSODE does not inhibit lipoxygenase. Therefore, the loss of activity in Figure 1 must be due to inactivation that occurred at the higher concentrations in the incubation mixture and was not reversed by dilution. The loss of activity was not simply due to reduction of the iron from Fe(III) to Fe(II), since the assay mixture contained 13-HPOD, which would reoxidize the iron. No activity was restored when enzyme that had been inactivated by S-12-HSODE was dialyzed at 3 °C for 1−24 h against either 20 mM phosphate, pH 7.0, or 50 mM borate, pH 9.0. These observations indicate that the inactivation by S-12-HSODE is irreversible.

No inactivation was detectable when S-12-HSODE was incubated with lipoxygenase in the absence of 13-HPOD. This observation suggests that the ferric enzyme is the target of the inactivator. As shown in Table 1, the extent of inactivation increases with increasing 13-HPOD up to about 2 μM. These results imply that maximal inactivation requires a concentration of 13-HPOD that is 20% of the S-12-HSODE concentration.

The results in Figure 1, as well as additional data at other concentrations (Table 2), indicate that the extent of inactiva-

Table 1: Dependence of Lipoxygenase Inactivation by S-12-HSODE on 13-HPOD[a]

[13-HPOD] (μM)	% inactivation[b]
0	0
0.2	9
0.5	26
1.0	52
2.0	75
5.0	69

[a] Each experiment contained 0.2 μM lipoxygenase and 10 μM S-12-HSODE in 50 mM borate, pH 9.0, at 25 °C. [b] The percentage of activity lost after 16−20 min relative to a control from which S-12-HSODE was omitted.

Table 2: Inactivation of Lipoxygenase by Thiols[a]

thiol	thiol concn (μM)	13-HPOD concn (μM)	% inactivation[b]	inactivation stoichiometry[c]
S-12-HSODE	4.0	2.0	31	65
	5.0	2.0	39	64
	8.0	2.0	66	61
	10	2.0	77	65
R-12-HSODE	10	10	22	225
	25	25	48	260
12-HSODA	1.5	10	48	16
	3.0	10	77	19
	3.0	3.0	74	20
1-octanethiol	4.0	10	42	48
	8.0	10	84	48

[a] Each experiment contained 0.2 μM lipoxygenase in 50 mM borate, pH 9.0, at 25 °C. [b] Refers to the percentage of activity lost after 16−20 min relative to a control from which thiol was omitted. [c] The molar ratio of thiol to inactivated enzyme. This value is calculated by dividing the thiol concentration by the product of the enzyme concentration and the percent inactivation after 16−20 min. The calculated value corresponds to the molar excess of thiol to enzyme that would be expected to cause complete inactivation.

tion after 16−20 min is proportional to the S-12-HSODE concentration. Extrapolation of the data implies that 60−65 equiv of S-12-HSODE would be required for complete inactivation of the enzyme.

Ferric Lipoxygenase Catalyzes Oxidation of the Thiol Group S-12-HSODE. The stoichiometry of inactivation by S-12-HSODE is consistent with the hypothesis that lipoxygenase catalyzes a reaction on S-12-HSODE and that this reaction leads to irreversible inactivation on 1 out of 60−65 turnovers. To determine whether lipoxygenase catalyzes a reaction on the thiol group of S-12-HSODE, 2.0 μM lipoxygenase was incubated with 50 μM S-12-HSODE and 20 μM 13-HPOD in 50 mM borate, pH 9.0, and the thiol concentration was monitored with DTNB (*30*) as described under Materials and Methods. After 2 min, the observed thiol concentration was 11 μM, of which 10 μM was due to the enzyme, as determined by a control experiment from which S-12-HSODE was omitted. The data imply that essentially all of the thiol of S-12-HSODE was consumed. No thiol consumption was detected if enzyme was omitted.

Oxygen electrode measurements indicated that addition of 50 μM S-12-HSODE to 2 μM lipoxygenase and 20 μM 13-HPOD in 50 mM borate, pH 9.0, led to consumption of 45 ± 8 μM O_2. No significant oxygen consumption was detected when enzyme or 13-HPOD was omitted.

The foregoing results demonstrate that ferric lipoxygenase catalyzes a reaction on S-12-HSODE that consumes the thiol group and approximately 1 equiv of O_2. Analysis of the products of this reaction by HPLC and TLC indicates that the product mixture is complex and that none of the products absorbs significantly in the UV. TLC with iodine-vapor detection revealed at least six products, all of which are more polar than S-12-HSODE.

When 125 μM S-12-HSODE was incubated with 0.5 μM lipoxygenase and 25 μM 13-HPOD, the initial rate of oxygen consumption was 28.5 μM/min. The rate of oxygen uptake was reduced to 3.1 μM/min in a parallel experiment using lipoxygenase that had been inactivated by ETYA (*31*) to 9% of its original catalytic activity. Experiments with DTNB indicate that the ETYA treatment reduced the rate of consumption of the thiol group of S-12-HSODE to 10−20% of that observed with untreated enzyme. These results demonstrate that enzyme that has lost its normal catalytic activity by inactivation with ETYA has also lost the ability to catalyze the oxidation of S-12-HSODE.

Thiol Specificity. R-12-HSODE inactivates lipoxygenase in a manner similar to the *S* isomer, but approximately 2.5-fold higher concentrations of the *R* enantiomer are required for inactivation (see Table 2). The data imply that more than a 200-fold excess of R-12-HSODE over enzyme would be required for complete inactivation. With the *R* isomer, as with the *S*, most of the loss of activity occurs in the first few minutes, and activity is not recovered by dilution or dialysis.

Incubation of R-12-HSODE with lipoxygenase in the presence of 13-HPOD results in oxygen consumption. When 50 μM R-12-HSODE was incubated with 0.25 μM lipoxygenase in the presence of 50 μM 13-HPOD, the initial rate of oxygen consumption was 67 ± 6 μM/min. In an identical experiment with S-12-HSODE, the initial rate was 39 ± 6 μM/min. Thus, R-12-HSODE appears to be oxidized more rapidly than S-12-HSODE, but the *R* isomer inactivates on a lower fraction of turnovers (1 in 225−260) than the *S* (1 in 60−65). The initial rates were measured over the first 10% of the reaction, which corresponds to about 20 turnovers. Less than 10% of the enzyme should be inactivated during the first 20 turnovers of R-12-HSODE, and about 30% of the enzyme should be inactivated during the first 20 turnovers of the *S* isomer. These considerations imply that more efficient inactivation by S-12-HSODE may account for some but not all of the observed difference in initial rates of oxidation of the two enantiomers.

Rapid irreversible inactivation was also observed with racemic 12-mercaptooctadecanoic acid (12-HSODA), the saturated analogue of 12-HSODE. Interestingly, this substance is more effective as an inactivator than either enantiomer of 12-HSODE. The data imply that slightly less than a 20-fold excess of 12-HSODA is required to completely inactivate the enzyme (Table 2). Oxygen consumption can be observed when 12-HSODA is incubated with lipoxygenase and 13-HPOD. The rate of oxygen uptake decreases rapidly with time due to inactivation of the enzyme, so it is not possible to estimate an initial rate that can be compared with those for the two enantiomers of 12-HSODE. The results with 12-HSODA indicate that a C_9-C_{10} double bond, which is present in linoleic acid, is not required for the action of lipoxygenase on thiol-containing fatty acids.

The observation of inactivation with 12-HSODA led us to consider the possibility that lipoxygenase inactivation might be a general property of hydrophobic thiols.

1-Octanethiol was found to be a potent inactivator, and activity was not restored by dilution or dialysis. When 1-octanethiol was incubated with lipoxygenase in the presence of 13-HPOD, oxygen consumption occurred at about 40% of the rate observed with S-12-HSODE. The data in Table 2 imply that a 48-fold excess of this compound is required for complete inactivation and suggest that inactivation occurs on 1 of 48 turnovers. Thus, 1-octanethiol is oxidized more slowly than S-12-HSODE, but 1-octanethiol inactivates slightly more efficiently than S-12-HSODE.

No inactivation was detected when 0.2 μM lipoxygenase (in the presence of 20 μM 13-HPOD) was incubated for 20 min with each of the following thiols (at 100 μM): mercaptoethanol, dithiothreitol, L-cysteine, glutathione, *N*-acetylcysteamine and (2*S*)-*N*-(3-mercapto-2-methylpropionyl)-L-proline (captopril). Each of these compounds was also tested for the ability to elicit O_2 consumption in the presence of 2 μM lipoxygenase plus 20 μM 13-HPOD, and no activity was detected in any case. These results imply that hydrophobicity is a crucial requirement for the inactivation of lipoxygenase by thiols and for the oxidation of these substances by the enzyme.

Lipoxygenase-Mediated Conversion of 1- Octanethiol to 1-Octanesulfonic Acid. 1-Octanethiol (50 μM) was incubated with 4 μM lipoxygenase in 50 μM borate, pH 9.0, at 3 °C as described under Materials and Methods.[2] After 2 min, the reaction mixture was acidified, and the products were recovered by solid-phase extraction and treated with diazomethane. GC of the methylated products from the enzymatic reaction revealed a peak at 7.75 min that was not present in controls from which enzyme was omitted. A peak with essentially the same retention time (7.78 min) was obtained on a sample of methyl 1-octanesulfonate prepared by treatment of 1-octanesulfonic acid with diazomethane. The EI mass spectrum of the enzymatic product (Figure 2A) was identical with that of methyl 1-octanesulfonate (Figure 2B), and the CI spectra of both substances gave the expected M+1 peak for methyl 1-octanesulfonate at *m*/*z* 209. These results demonstrate that lipoxygenase-mediated oxygenation of 1-octanethiol produces 1-octanesulfonic acid. Additional weak peaks were detectable in the gas chromatogram of the methylated products from the enzymatic reaction, but these peaks were also present in a control from which enzyme was omitted. Since 1-octanesulfinic acid was thought to be a possible product of the enzymatic reaction, GC/MS of the methylated products was carried out using CI with specific-ion monitoring at *m*/*z* 193 (expected M+1 for methyl 1-octanesulfinate), but no peaks were detected.

DISCUSSION

Our results demonstrate that soybean lipoxygenase-1 is irreversibly inactivated by micromolar concentrations of hydrophobic thiols. Lipoxygenase also catalyzes the oxidation of the sulfhydryl group in these inactivators. In the case of 1-octanethiol, the oxidation product was identified as 1-octanesulfonic acid. To our knowledge, this is the first reported example of a lipoxygenase-catalyzed conversion of divalent sulfur to an oxygenated derivative.

With the thiols investigated in this work, neither inactivation nor oxidation occurred in the absence of 13-HPOD. These findings suggest that the ferric form of the enzyme is required for these processes. A likely initial chemical step is the one-electron oxidation of the thiol by the ferric ion to generate the ferrous form of the enzyme, a proton, and a thiyl radical, **5** (Scheme 2) (*32*, *33*). Chemical precedence suggests that the thiyl radical would react with O_2, to generate a thiylperoxy radical, **6** (*33*, *34*). This reaction has been well characterized in studies of thiyl radicals generated by pulse radiolysis (*35*−*38*), and the rate of the reaction is close to the diffusion limit in several cases (*37*−*39*). In aqueous solution, thiylperoxy radicals undergo a complex and incompletely understood series of reactions that leads ultimately to sulfonic acids, disulfides, and additional minor products, with the yield of sulfonic acid increasing as oxygen concentration increases and the thiol concentration decreases (*37*). Sulfonic acids are also formed from thiyl radicals generated photolytically in the presence of oxygen (*40*) and by autoxidation of thiols under alkaline conditions (*41*). One possible pathway to sulfonic acids is the isomerization of thiylperoxy radicals to sulfoxyl radicals, $RSO_2\cdot$, which can react further with oxygen to give sulfinylperoxyl radicals, $RSO_2OO\cdot$ (*34*, *35*, *37*). On the basis of this nonenzymatic chemistry, we suggest that the 1-octanesulfonic acid formed in the lipoxygenase-catalyzed oxygenation of 1-octanethiol is initiated by the two steps depicted in Scheme 2.

It is likely that the action of lipoxygenase on the other active thiols in this study is also initiated by the chemistry in Scheme 2. In the case of S-12-HSODE, the reaction leads to a complex mixture of products that have not been characterized. As noted above, there is chemical precedence for formation of disulfides and other products from thiylperoxy radicals (*37*). With S-12-HSODE, additional products may result from the ability of thiyl radicals to react with carbon−carbon double bonds (*42*−*44*). The thiyl radical derived from S-12-HSODE could react with the double bond in this compound to give cyclic carbon radicals, which might subsequently react with oxygen.

The first two steps in Scheme 2 are analogous to the first two steps in the radical-based mechanism that has been proposed for the normal oxygenation reaction catalyzed by lipoxygenase (*12*, *45*); the only difference is that the initial intermediate is a thiyl radical rather than a delocalized carbon radical. It has been proposed that a ferric hydroxide moiety catalyzes the initial hydrogen abstraction in the case of normal substrates (*46*−*48*), and this mechanism may also be operative in the case of thiols.

In light of the above mechanistic considerations, it is noteworthy that lipoxygenase that was inactivated with ETYA lost its ability to oxidize S-12-HSODE to about the same extent as it lost the ability to oxygenate linoleic acid. This result implies that oxidation of thiols by lipoxygenase requires the normal catalytic capability of the enzyme. ETYA is an irreversible, mechanism-based inactivator of lipoxygenase, but inactivation is not caused by covalent attachment of the carbon skeleton of the inactivator (*31*, *49*). Thus, the active site is not blocked by treatment with ETYA; presumably it is modified in a way that prevents the processing of normal substrates. Our findings indicate that this modification also prevents processing of S-12-HSODE.

[2] Control experiments demonstrated that nonezymatic loss of the thiol group was negligible at 50 μM 1-octanethiol but became significant at higher concentrations.

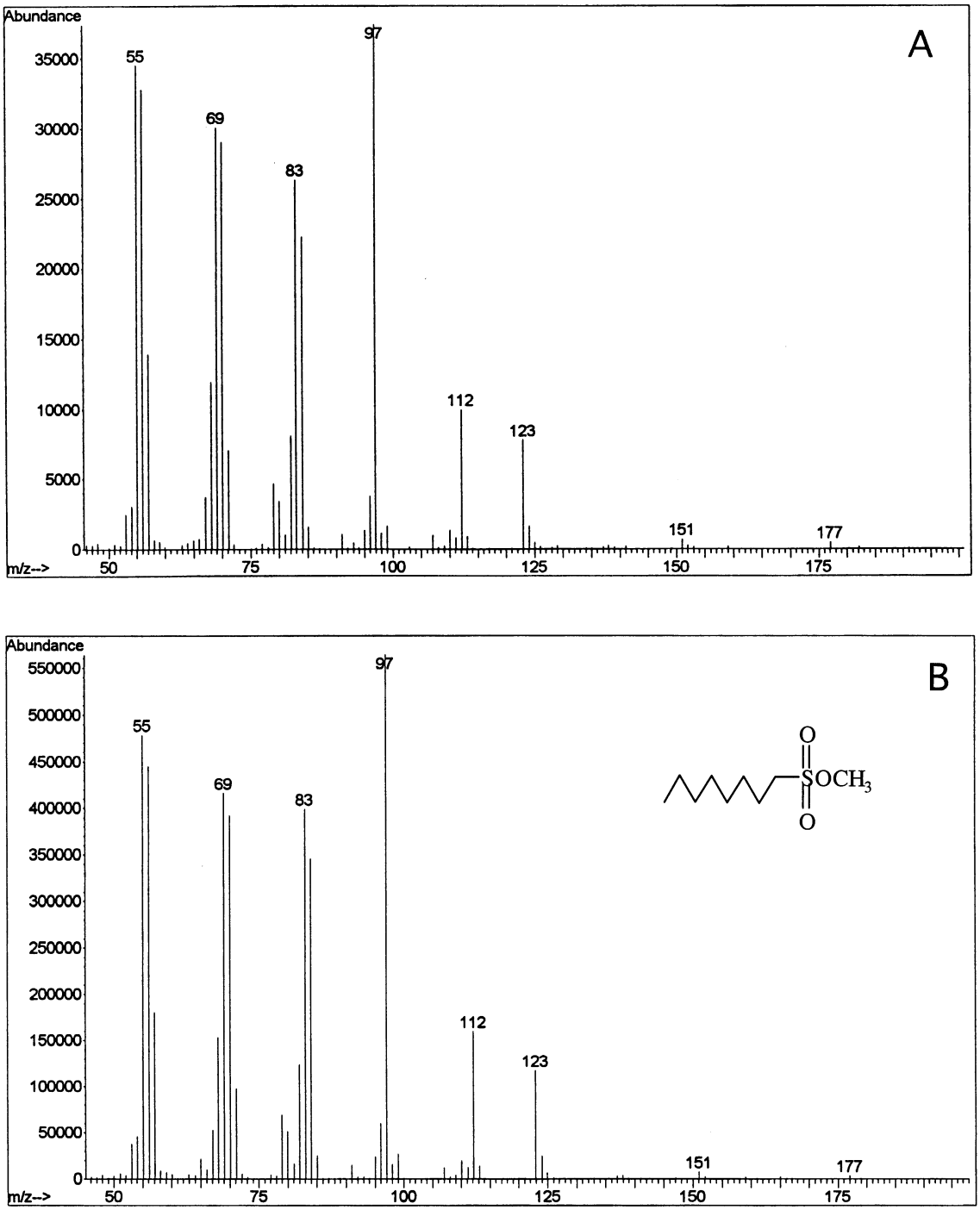
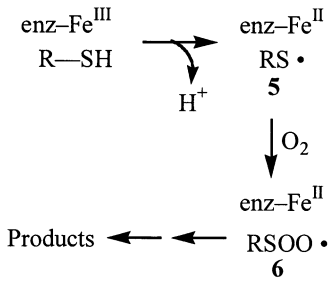


FIGURE 2: (A) EI mass spectrum of the methyl ester of the product from the oxidation of 1-octanethiol by lipoxygenase. (B) EI mass spectrum of methyl 1-octanesulfonate.

Scheme 2



On the basis of our present results, we cannot exclude the possibility that lipoxygenase activates O_2 to a superoxo species or some other reactive oxidant which oxidizes thiol groups. However, past attempts to detect binding or activation of O_2 by lipoxygenase have been unsuccessful (*50*, *51*). Mechanistic studies on the normal lipoxygenase reaction strongly support the notion that the enzyme activates substrates to react with O_2 (*12*, *50*), and the pathway in Scheme 2 follows this pattern.

Whatever its detailed mechanism, the oxidation of the thiol group by lipoxygenase almost certainly involves reactive intermediates—radicals and/or peroxides. Inactivation likely involves reaction of one of these intermediates with a functional group at the active site. Elucidation of the pathway

to inactivation will require additional studies by EPR and mass spectrometry.

Inhibition of lipoxygenase by 1-octanethiol and other simple aliphatic thiols has been reported previously (*52*). The inhibition was treated as reversible and found to be noncompetitive with substrate. The possibility that the thiols were being oxidized was not investigated. Many years ago, Mitsuda et al. (*53*) reported that lipoxygenase is irreversibly inactivated by prolonged incubation (12 h) of lipoxygenase with 100 μM L-cysteine under aerobic conditions. The inactivation was blocked by catalase and was therefore attributed to hydrogen peroxide formed by autoxidation of L-cysteine. Catalase-sensitive inactivation of arachidonate-5-lipoxygenase by dithiothreitol has also been reported (*54*). The inactivation reported here for S- and R-12-HSODE, 12-HSODA, and 1-octanethiol was not blocked by catalase at concentrations (200 μg/mL) that are 10-fold higher than that which blocks inactivation of 5-lipoxygenase by dithiothreitol. In addition, the inactivation observed with the active thiols in this study occurs much more rapidly than in published cases involving hydrogen peroxide formation. We propose that the inactivation reported here occurs by a previously unrecognized mechanism involving redox chemistry between the thiol group of the inactivator and the ferric ion in lipoxygenase.

The amount of 13-HPOD required for optimal inactivation by the thiols in this study is greater than stoichiometric with enzyme but less than stoichiometric with substrate. In the case of S-12-HSODE, maximal inactivation required about 0.2 mol of 13-HPOD per mole of inactivator (Table 1). A possible explanation for this stoichiometry is that processing of S-12-HSODE by ferric lipoxygenase involves reduction of the active-site iron to the ferrous state with subsequent reoxidation on about 80% of turnovers; on the other 20% of turnovers, an intermediate dissociates from the enzyme while the iron is in the ferrous form, so that 13-HPOD is required to reoxidize the enzyme. A similar scenario has been shown to occur during the inactivation of lipoxygenase by 12-IODE (*21*).

Our initial hypothesis that S- and/or R-12-HSODE might be inactivators of lipoxygenase was based on the structural resemblance of these substances to 12-IODE. The discovery that 12-HSODA and 1-octanethiol are good inactivators indicates that the resemblance to 12-IODE is much less important than we expected. The broad specificity exhibited by thiols as lipoxygenase inactivators is in sharp contrast to the highly specific nature of the inactivation by 12-IODE. Neither the saturated analogue of 12-IODE (*19*) nor 1-iodooctane[3] shows any activity as an irreversible inactivator of lipoxygenase. The source of this stringent specificity has been revealed by the observation of a kinetic isotope effect on inactivation by 11,11-dideuterio-12-IODE, which demonstrates that inactivation by 12-IODE is triggered by cleavage of the C_{11}−H bond (*21*). The observation of inactivation with 12-HSODA and 1-octanethiol argues against such a mechanism for inactivation by 12-HSODE and is consistent with a mechanism that involves redox chemistry at the thiol group.

Experiments with S- and R-12-HSODE indicate that the effects that we observe exhibit only modest stereoselectivity. The slightly higher rate of oxygen consumption observed with the *R* enantiomer suggests that the thiol group of the *R* isomer may be able to bind closer to the ferric ion than is the case with the *S* enantiomer. The lower efficiency of R-12-HSODE as an inactivator must result from a slightly reduced frequency at which an intermediate or product reacts with the enzyme.

The initial rate of oxygen consumption that occurs when 1-octanethiol is incubated with lipoxygenase (in the presence of 13-HPOD) is 2.5 times lower than that observed with S-12-HSODE and about 5 times lower than that observed with R-12-HSODE. These differences may be due to greater structural resemblance of S- and R-12-HSODE to normal lipoxygenase substrates, which increases the affinity for these substances for the active site. Even if this is true, the differences are small and suggest that that neither enantiomer of 12-HSODE is an optimized structure in which efficient oxidation is facilitated by efficient binding of the thiol group in close proximity to the iron.

Neither inactivation nor oxygen uptake was observed with hydrophilic thiols such as L-cysteine, mercaptoethanol, dithiothreitol, or glutathione.[4] These substances probably have low affinity for the lipoxygenase active site, which is relatively hydrophobic. Two thiols of intermediate polarity, *N*-acetylcysteamine and the antihypertensive drug captopril, also failed to inactivate the enzyme.

Our results demonstrate that hydrophobic thiols rapidly inactivate soybean lipoxygenase by an oxidative mechanism. Hydrophobicity appears to be a critical requirement, but close resemblance to lipoxygenase substrates is not. Lipoxygenases are widespread in the plant and animal kingdoms, and the vulnerability of these enzymes to inactivation by hydrophobic thiols may have significant environmental and toxicological consequences.

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[3] A. M. Grandizio and C. H. Clapp, unpublished experiments.

[4] At concentrations (0.5−3 mM) higher than those used in this study, glutathione has been shown to undergo oxidation to the corresponding disulfide when incubated with lipoxygenase in the presence of linoleic acid (*55*). 13-HPOD and H_2O_2 can substitute for linoleic acid in this cooxidation but are considerably less effective. Reaction between bound hydroperoxide radicals and glutathione was suggested as the major pathway of glutathione oxidation under these conditions.

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