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Analysis of a Specific Oxygenation Reaction of Soybean Lipoxygenase-1 with Fatty Acids Esterified in Phospholipids†

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ABSTRACT: Soybean lipoxygenase was reacted with phosphatidylcholine (at pH 9, with 10 mM deoxycholate), and the oxygenation products were analyzed by high-pressure liquid chromatography, UV, gas chromatography–mass spectrometry (GC–MS), and NMR. The structures of the intact glycerolipid products were established by GC–MS of diglycerides recovered by phospholipase C hydrolysis and by proton NMR of the intact phosphatidylcholine. These analyses, together with analyses of the transesterified fatty acids, indicated that arachidonyl and linoleoyl moieties in the phosphatidylcholine were converted exclusively to the 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate analogues, respectively. Control experiments proved that the intact phospholipid (and not hydrolyzed/reesterified fatty acid) was the true substrate of the oxygenation reaction. Phosphatidylethanolamine and phosphatidylinositol lipids were also substrates for specific oxygenation by the soybean lipoxygenase. The results provide concrete evidence that fatty acids esterified in phospholipid can be subject to highly specific oxygenation by a lipoxygenase enzyme.

There are two main isoenzymes in soybeans, and these are classified on the basis of their substrate specificities, pH optima, and the specificity of the oxygenation reaction they catalyze with polyunsaturated lipids. The classic enzyme originally isolated by Theorell, Holman, and Akeson (1947) is now called soybean lipoxygenase-1. This enzyme is known to react with polyunsaturated fatty acids with optimal activity around pH 9. The enzyme is quite discriminating in terms of the range of acceptable fatty acid substrates, and characteristically, a single major hydroperoxide is formed as product (Hamberg & Samuelsson, 1966; Holman et al., 1969). Typically, the hydroperoxide group is introduced into the free fatty acid substrate in the ω6 position and in the Ls steric configuration (Hamberg & Samuelsson, 1967). By contrast, the soybean lipoxygenase-2 will react with free acid or ester substrates with the optimal activity being around pH 6.5. This isoenzyme will accept a wide range of polyunsaturated lipid substrates, and it will catalyze oxygenation with little or no positional and stereo specificity. In effect, the soybean lipoxygenase-2 is a catalyst of enzyme induced autoxidation of polyunsaturated lipids. Although there are several reports of oxygenation of esterified polyunsaturated lipids by soybean lipoxygenase(s) [e.g., Koch et al. (1958), Guss et al. (1968), and Christopher et al. (1970)], these would generally be considered as reactions of the nonspecific soybean lipoxygenase-2 isoenzyme. Invariably, the reactions that have been subjected to product analysis have been shown to be lacking in specificity of oxygenation [e.g., Christopher et al. (1972) and Roza and Francke (1978)].

The dogma stemming from these findings is that positional-specific and stereo-specific oxygenation is associated with reaction of a lipoxygenase and a free acid substrate. However, this paper is concerned with establishing that this need not be the case. A recent report by Eskola and Laasko (1983)

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showed that soybean lipoxygenase-1 could catalyze the oxygenation of polyunsaturated fatty acids esterified in phosphatidylcholine. Reaction was dependent on solubilization of the phospholipid with bile salts; with other detergents, there was no reaction. Because oxygenation was catalyzed by soybean lipoxygenase-1 acting at pH 9 and because reaction showed a critical dependence on the micellar structure of the detergent, it seemed that this reaction might be more specific than a random "enzyme-catalyzed autoxidation" of the esterified substrate. Accordingly, this bile salt dependent reaction of phosphatidylcholine with soybean lipoxygenase was selected for detailed study. In this paper the structural analysis of the products of this reaction are reported. The results establish the precedent for the highly specific oxygenation of an ester substrate by a lipoxygenase enzyme.

Materials and Methods

Materials. Soybean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) was the type 1 (130000 units/mg of protein) obtained from Sigma Chemical Co., St. Louis, MO. Sigma also supplied the following phospholipids and phospholipases: L-3-phosphatidylcholine (from egg yolk), L-3-phosphatidylcholine (1-palmitoyl-2-linoleoyl), L-3-lysophosphatidylcholine (1-palmitoyl), phospholipase A2 (from *Naja naja* venom), and phospholipase C (type V, from *Bacillus cereus*). Single molecular species of L-3-phosphatidylcholine (1-palmitoyl-2-arachidonyl and also 1-stearoyl-2-arachidonyl) were purchased from Avanti Polar Lipids, Birmingham, AL.

Radiolabeled phospholipid standards were obtained from New England Nuclear, Boston, MA (1-palmitoyl-2-[1-^{14}C]-arachidonyl-L-3-phosphatidylcholine and (1-palmitoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylethanolamine) and also from the Amersham Corp., Arlington Heights, IL (1-palmitoyl-2-[^{14}C]linoleoyl-L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine, 1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl-L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]linoleoyl-L-3-phosphatidylethanolamine, 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylinositol, and 1,2-dipalmitoyl[*N-methyl*-^{14}C]-L-3-phosphatidylcholine.

The 2-palmitoyl ester of [*N-methyl*-^{14}C]PC[1] was cleaved with phospholipase A_2 (Dennis, 1973) and replaced (Lands & Hart, 1965) with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (obtained from Amersham). The resulting product was purified by RP-HPLC essentially as described below, giving a radiolabeled phosphatidylcholine with ^{14}C in the choline moiety and ^{3}H in the 2-arachidonyl ester. A radiolabeled oxygenated phosphatidylcholine standard was prepared by the same means, in which [5,6,8,9,11,12,14,15-^{3}H]-5-HETE [synthesized as described before (Maas et al., 1983] was coupled into the 2-position of L-3-lysophophosphatidylcholine.

Racemic hydroxyoctadecadienoates and hydroxyeicosatetraenoates were prepared by controlled autoxidation of the methyl esters in the presence of α-tocopherol (Peers & Coxon, 1983). The 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acids were prepared by reaction of the parent free acids with soybean lipoxygenase, and the corresponding hydroxy acids were obtained by triphenylphosphine reduction.

Common salts were obtained from Fisher Scientific Co., Fairlawn, NJ, sodium deoxycholate was from Sigma, and all solvents were of distilled-in-glass grade from American Burdick & Jackson, Muskegon, MI. *N,N*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco, Bellefonte, PA. The nonadeuteriated ($^{2}H_9$) BSTFA and the *tert*-butyldimethylsilyl-BSTFA analogues were purchased from Regis Chemical Co., Morton Grove, IL. Sodium methoxide was prepared by reaction of sodium metal with methanol. Oxygen-18-labeled water (96% $H_2{}^{18}O$) was supplied by the Monsanto Corp., Mound Facility, Miamisburg, OH.

Reaction of Phospholipids with Lipoxygenase. Incubation conditions were essentially as described by Eskola and Laakso (1983): 1 mM phospholipid was solubilized in 0.2 M pH 9 borate buffer containing 10 mM deoxycholate and reacted with 50 μg/mL soybean lipoxygenase at room temperature for 30 min. Reaction was monitored on a Gilson oxygraph with a 2-mL chamber; the oxygraph was calibrated by recording the oxygenation of known amounts of arachidonic acid with dilute soybean lipoxygenase (1 μg/mL) in deoxycholate-free buffer. The reaction of phospholipid was also monitored by UV spectroscopy of dilutions of the mixture. With the arachidonyl-containing PE and PI there was insufficient substrate available for either of these approaches; these reactions were conducted in a total volume of 50 μL and subsequently analyzed by direct injection into the RP-HPLC.

Extraction and HPLC of Phospholipids. Phospholipids were extracted according to the procedure of Bligh and Dyer (1959) or, alternatively, by use of 500-mg C18 Bond-Elute cartridges (Analytichem International, Harbor City, CA), in which case the samples were applied directly in the borate buffer, the column was washed with water, and the phospholipids were eluted with methanol. Reversed-phase HPLC was carried out with very minor changes to the method described by Patton et al. (1981); the column was a 5-μm Ultrasphere (4 × 250 mm) from Beckman Instruments Inc., Irvine, CA, the solvent system was water/methanol/acetonitrile (70:930:50, by volume) containing 20 mM choline chloride, and the flow rate was 2 mL/min at room temperature. The effluent was monitored by UV at 235 nm for detection of oxygenated products and at 205 nm for polyunsaturated substrates. Normal-phase HPLC was carried out with a μPorasil column (4 × 250 mm) from Waters Associates (Milford, MA) in a solvent system of hexane/2-propanol/water (4:6:1, by volume) with elution at 1 mL/min and UV detection at 235 or 205 nm as above.

Isolation and HPLC of Fatty Acids and Diglycerides. These reactions were carried out after chromatography of the intact phospholipids. Transesterification was accomplished by dissolving the phospholipid in 50 μL of methanolic sodium methoxide. Reaction was allowed to proceed for 10 min at room temperature under argon, then 325 μL of 0.1 N HCl was added, and the solution was immediately extracted with 2 volumes of hexane. After a second hexane extraction, the combined organic phases were washed once with an equal volume of water and evaporated to dryness. Phospholipase A_2 hydrolysis was carried out as described by Dennis (1973). Phospholipase C hydrolysis was conducted exactly as described by Majerus and Prescott (1982). For RP-HPLC of methyl esters, the column was the 5-μm Ultrasphere (4 × 250 mm) and the solvent system methanol/water (80:20, by volume); for chromatography of free acids, the solvent was modified to methanol/water/acetic acid in the proportions 75:25:0.01 (by volume). Methyl esters and diglycerides were run on normal-phase HPLC with a 5-μm silica column (4.6 × 250

[1] Abbreviations: RP-HPLC, reversed-phase high-pressure liquid chromatography; SP-HPLC, straight-phase high-pressure liquid chromatography; GC–MS, gas chromatography–mass spectrometry; amu, atomic mass units; COSY, correlation spectroscopy; PC, PE, and PI, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; MeOH, methanol; HAc, glacial acetic acid.

mm) from Alltech Associates, Norcross, GA, under a solvent system of hexane/2-propanol in the volumetric proportions of 100:0.5 and 100:3, respectively.

Ultraviolet Spectroscopy. The instrument was a DU-7 scanning UV/vis spectrophotometer, and calibration was checked with a holmium oxide filter using the absorbance peak at 279 nm (λ_{max} observed at 297.2 nm). Spectra were recorded in the enzyme incubation buffer or in methanol. It was observed that, in attempting to monitor the enzyme reactions with substrate concentrations of 1 mM, there was a distinct problem with saturation of the absorbance readings. When this was due to the high concentration of conjugated diene containing products, the problem could be circumvented by dilution of the sample. However, it was noticed early in these investigations that "old" samples of phospholipid were sometimes autoxidized to such an extent that the lower UV was saturated even before the reaction began; this low transmittance was associated with nonlinearity in the absorbance changes measured during the reaction. Undoubtedly, this artifact accounts for the supposed nonequivalence of oxygen uptake and formation of diene-containing products (and the apparent shift in λ_{max}) in the original description of this soybean lipoxygenase reaction (Eskola & Laakso, 1983).

NMR Spectroscopy. Proton NMR spectra were recorded with a Bruker AM 400 (400-MHz) instrument (in chloroform with tetramethylsilane as reference) with 16 000 data points zero filled to 32 000, sweep width 5000 Hz, giving 0.3 Hz/point; 300 scans were taken, and a small amount of resolution enhancement was used in sample workup. The two-dimensional (2-D) spectrum was run on an IBM/Bruker NS300 (300-MHz) instrument; 256 1K spectra were collected by the Bruker standard COSY microprogram with a $90°-\tau-60°$ sequence of pulses. Sine-bell apidization, magnitude calculation, and symmetrization with employed; the data in F1 were zero filled once prior to transformation. The quantity of sample was approximately 1.5 μmol of phospholipid.

Derivatization for GC–MS. Methyl esters were prepared by dissolving the sample in 50 μL of methanol followed by addition of a few drops of ethereal diazomethane; reaction was terminated by evaporation to dryness after 2 min at room temperature. Trimethylsilyl ethers were prepared by reaction in 10 μL each of pyridine and BSTFA at room temperature for 60 min. *tert*-Butyldimethylsilyl ethers were formed in the same way with the *tert*-butyldimethylsilyl analogue of BSTFA.

Gas Chromatography–Mass Spectrometry. GC–MS analyses were performed on a Nermag R10-10C quadrapole instrument operated in the electron impact mode. The gas chromatograph was equipped with a 10-m DB-1 capillary column (0.25-mm internal diameter, coating thickness 0.25 μm); samples were injected in dodecane with the oven temperature at 190 °C, and after 1 min the temperature was programmed at 10 deg/min. Mass spectra were recorded with an electron energy of 70 eV.

Steric Analysis of Alcohol Configuration. Two methods were used. The method of Hamberg (1971) was carried out as previously described (Maas et al., 1982); this involves formation of the (−)-menthoxycarbonyl derivative of the methyl ester of the hydroxy fatty acid, oxidative ozonolysis, reesterification of the resulting acid fragment, and analysis of the *R* and *S* forms by GC–MS/selected ion monitoring vs. the corresponding authentic racemic derivatives. For ω6 fatty acids oxygenated in the ω6 position, the ozonolysis fragment analyzed is the methyl ester (−)-menthoxycarbonyl derivative of 2-hydroxyheptanoic acid. Authentic *S* and *RS* standards were prepared from 15(*S*)- and 15(*RS*)-HETE.

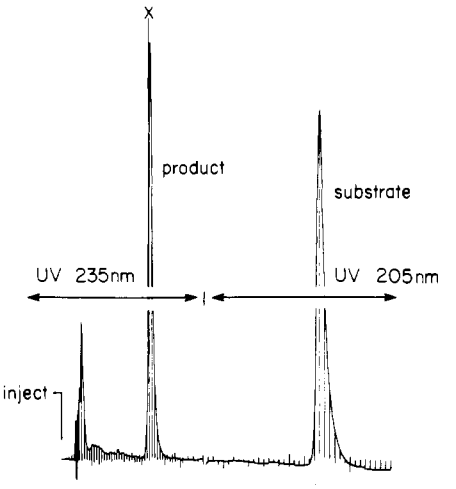

FIGURE 1: Reversed-phase HPLC analysis of reaction of 1-palmitoyl-2-arachidonylphosphatidylcholine with soybean lipoxygenase. Column: 5-μm ODS Ultrasphere (Altex). Solvent: MeOH/CH_3CN/H_2O (90:6:4, v/v/v) containing 20 mM choline chloride. Flow rate: 2 mL/min. Detection: UV at 235 nm (at peak of product, "X" indicates sensitivity cut in half) and at 205 nm to detect unreacted substrate. Elution volume of product = 18 mL and of substrate = 52 mL.

In addition, an HPLC method was used; this is based on resolution of the carbamate diastereomers formed by reaction of the methyl ester of the alcohol with the isocyanate derivative of dehydroabietylamine (Gottstein & Cheney, 1965; Corey & Hashimoto, 1981; Falck et al., 1984). The methyl ester of the hydroxy fatty acid (20 μg) was reacted with the isocyanate (1 mg), together with 4-(dimethylamino)pyridine catalyst (0.75 mg), in 25 μL of dichloromethane for about 3 days at room temperature under argon. The sample was evaporated dry and dissolved in hexane (leaving the reagents undissolved), and aliquots were injected on a normal-phase HPLC system [5-μm Alltech silica, 4.6 × 250 mm, hexane/2-propanol (100:0.2, by volume) at 0.5 mL/min, UV detection at 235 nm, and elution volume of products ≈ 40 mL].

RESULTS

Reaction of Phosphatidylcholine with Lipoxygenase. Under the standard reaction conditions, the initial rate of oxygen uptake with egg yolk PC was approximately 5 nmol/min. This is of the same order as reported by Eskola and Laakso (1983) and is approximately an order of magnitude slower than the rate of reaction of the free acid under the same conditions. With the single molecular species of 1-palmitoyl-2-linoleoyl-PC or 1-palmitoyl-2-arachidonyl-PC, the initial reaction rate was 2–3 times faster, as could be expected from the absence of the diluting effect of the disaturated fatty esters in the egg yolk PC. a quantitative comparison of micromoles of oxygen uptake with micromoles of conjugated diene formed gave a slight (15%) excess of UV-absorbing product; this slight discrepancy was caused by a small increase in the UV end absorption during the reaction.

HPLC of Products. Single molecular species of PC were each converted to a single more polar product, Figure 1. When egg yolk PC was the substrate, RP-HPLC analysis showed a pattern of more polar derivatives that were found to be the oxygenated derivatives corresponding to the original mixture of polyunsaturated esters of the PC. Thus, the major peaks were derived from 1-palmitoyl-2-linoleoyl-PC, 1-palmitoyl-2-arachidonyl-PC, 1-stearoyl-2-linoleoyl-PC, and 1-stearoyl-2-arachidonyl-PC (relative retention volumes of these products were 0.95, 1.00, 1.60, and 1.70, respectively) with many smaller peaks corresponding to the di-unsaturated

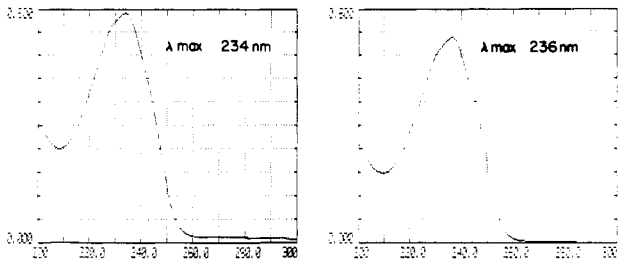

FIGURE 2: Ultraviolet spectra of oxygenated phospholipids. (Left) Product from soybean lipoxygenase reaction with 1-palmitoyl-2-linoleoyl-PC. (Right) Product from 1-palmitoyl-2-arachidonyl-PC. Spectra were recorded in methanol.

substrates and other species of PC.

Radiolabeled phospholipid substrates gave products that retained the label. When [*N-methyl*-^{14}C,^{3}H]arachidonyl-labeled phosphatidylcholine was the substrate, the single product retained both ^{14}C and ^{3}H; the ^{3}H molecules showed a slightly earlier elution on RP-HPLC, a property observed with both the starting material and the product.

On normal-phase HPLC the phospholipid products chromatographed as slightly more polar than their phosphatidylcholine parent; for example, the elution volumes of 1-palmitoyl-2-linoleoyl-PC and its oxygenation product were 16.5 and 18.5 mL, respectively, in a solvent of hexane/2-propanol/water (4:6:1, by volume).

UV Absorbance of Products. The products displayed a UV spectrum typical of a conjugated diene, Figure 2. Close scrutiny of the λ_{max} and the shape of the chromophore revealed some diagnostic features. Thus, the product from 1-palmitoyl-2-linoleoyl-PC had a chromophore that was identical in λ_{max} (233.5 nm) and shape to that of 9-hydroxy-10-*trans*-,12-*cis*- and 13-hydroxy-9*cis*,11-*trans*-linoleates. The UV spectrum of the product from 1-palmitoyl-2-arachidonyl-PC was indistinguishable from the spectrum of 5-HETE or 15-HETE (λ_{max} 236 nm) and different from that of 9-HETE/11-HETE (λ_{max} 1.5 nm lower wavelength and a more symmetrically shaped peak) or of 8-HETE/12-HETE (λ_{max} 0.5 nm higher).

Molar Content of Hydroperoxide. Quantitative triphenylphosphine reduction (Boeynaems et al., 1980; methyl eicosanoate as internal standard) was carried out on 0.1 μmol samples of RP-HPLC-purified phospholipid products. The result showed almost precisely (±2%) 1 mol of hydroperoxide/mol of conjugated diene. All further structural analyses were performed on the hydroxy derivative formed in this reaction.

Transesterification and Analysis of Oxygenated Fatty Acids. Transmethylation of the oxygenated phospholipid derived from 2-arachidonylphosphophatidylcholine yielded one major product that was identified by UV, HPLC, and GC–MS criteria as 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate (15-HETE) methyl ester. The same methods were used to confirm that the oxygenated linoleoyl derivative was methyl 13-hydroxy-9-*cis*,11-*trans*-octadecadienoate. Reversed-phase and normal-phase HPLC analyses indicated that the oxygenation reaction was more than 95% specific for the ω6 position of arachidonate and linoleate in phosphatidylcholine. The structure of the oxygenated glycerolipids was examined by NMR and GC–MS.

NMR Spectroscopy. The proton NMR spectra of 1-palmitoyl-2-arachidonyl-PC and of the corresponding soybean lipoxygenase product were recorded. The 400-MHz spectrum of the starting material showed essentially the same chemical shifts and multiplicities as reported by Murthy and Agranoff

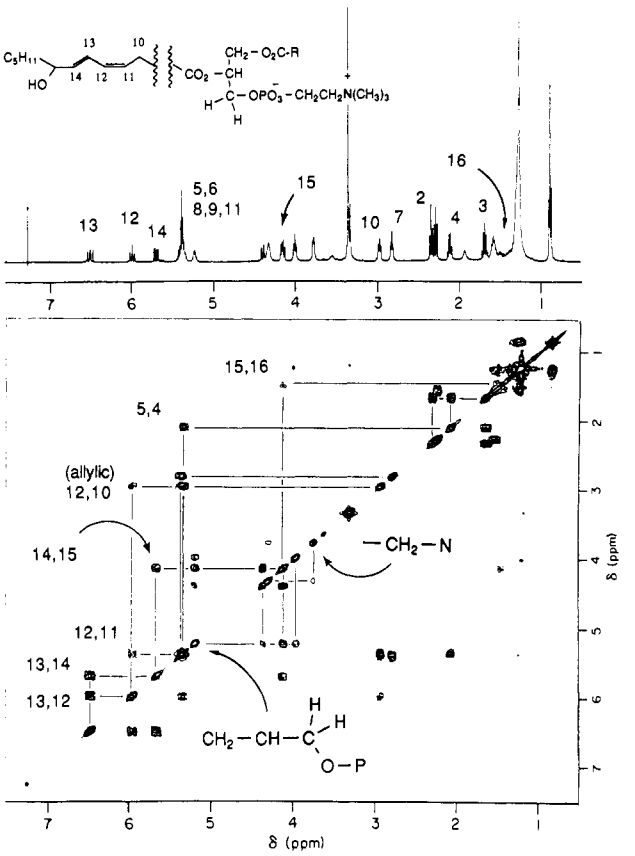

FIGURE 3: ^{1}H NMR spectrum (400 MHz) and two-dimensional COSY spectrum of 1-palmitoyl-2-(15-HETE)phosphatidylcholine. The protons of the 15-HETE component are numbered on the 1-D spectrum. The COSY was recorded separately on a Bruker 300-MHz instrument. The connectivities emphasized above the diagonal are from the 15-HETE component of the molecule. The connectivities emphasized below the diagonal are from the glycerol and choline protons.

(1982). Figure 3 shows the 400-MHz spectrum of the product and the corresponding two-dimensional COSY spectrum. Table I gives the chemical shifts, multiplicities, and key coupling constants. These analyses provide rigorous proof that the arachidonyl moiety of the PC substrate is replaced by a HETE moiety in the (triphenylphosphine-reduced) reaction product. In addition, the coupling constants of $J_{11,12}$ = 11 Hz and $J_{13,14}$ = 15 HZ confirm the cis/trans configuration of the conjugated diene in the 15-HETE.

Phospholipase C Hydrolysis/GC–MS of the Diglyceride. The soybean lipoxygenase product from 1-palmitoyl-2-linoleoyl-PC was cleaved with phospholipase C and the diglyceride purified by normal-phase HPLC; there was one major UV-absorbing peak accounting for 90% of the products and three more polar peaks of about 3% abundance each—these were not analyzed further. The UV chromophore of the major peak was identical with that of its phospholipid parent. An aliquot of the main peak was converted to the trimethylsilyl ether derivative and analyzed by GC–MS.

The mass spectrum, Figure 4, showed a molecular ion at *m/z* 752, corresponding to the bis(trimethylsilyl) ether of glycerol containing one saturated C_{16} acyl ester plus one diunsaturated hydroxy C_{18} acyl ester. This interpretation was supported by analysis of the hydrogenated analogue (M^+ absent, M − CH_3 at *m/z* 741), and of the corresponding analogues formed with deuteriated BSTFA, and of the *tert*-butyldimethylsilyl ether derivatives of both the native and the hydrogenated compound (M^+ absent in both, M − 57 at *m/z* 779 and 783, respectively). Structural assignment of the major

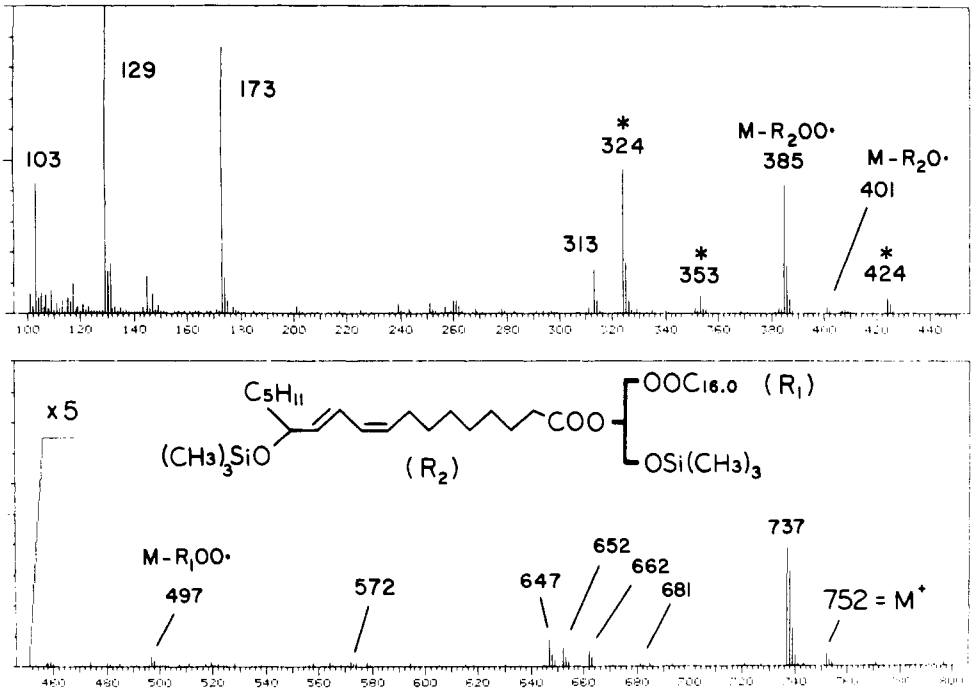

FIGURE 4: Mass spectrum of trimethylsilyl derivative of oxygenated diglyceride. The diglyceride was obtained by phospholipase C treatment of the product of reaction of soybean lipoxygenase with 1-palmitoyl-2-linoleoyl-PC. The spectrum was recorded under electron impact (70 eV). Note that ions are magnified times 5 above m/z 450. R_1 is the palmitate moiety ($C_{16.0}$).

Table I: ^{1}H NMR (400 MHz) of the Soybean Lipoxygenase Product from 1-Palmitoyl-2-arachidonylphosphatidylcholine

chemical shift (ppm)		proton(s)	coupling constants (Hz)
6.51	dd	H-13 (15-HETE), 1 H	$J_{12,13}$ = 11, $J_{13,14}$ = 16
6.00	t	H-12 (15-HETE), 1 H	11
5.71	dd	H-14 (15-HETE), 1 H	$J_{13,14}$ = 16, $J_{14,15}$ = 7
5.40	m	H-5, -6, -8, -9, -11 (15-HETE), 5 H	
5.24	m	C*H*OC, 1 H	
4.40	dd	CH_2OP, 1 H	J = 3, J_{gem} = 12
4.33	br m	POCH_2CH_2N, 2 H	
4.16	q	H-15 (15-HETE), 1 H	≈7
4.15	dd	CH_2OP, 1 H	J = 7, J_{gem} = 12
4.01	t	CH_2OC, 2 H	7
3.78	br t	CH_2N, 2 H	≈4–5
3.36	s	N(CH_3)$_3$, 9 H	
2.97	t	H-10 (15-HETE), 2 H	≈7–8
2.82	t	H-7 (15-HETE), 2 H	≈5–6
2.34	t	H-2 (15-HETE), 2 H	7
2.28	t	H-2 (palmitoyl), 2 H	7.5
2.12	q	H-4 (15-HETE), 2 H	7
1.69	p	H-3 (15-HETE), 2 H	7
1.58	br p	H-3 (palmitoyl), 2 H	
1.50	br m	H-16 (15-HETE), 2 H	
1.26	m	–CH_2–, 30 H	
0.89	t	CH_3, 6 H	7

ion fragments in Figure 4 was aided by reference to the reported fragmentation of derivatives of diacylglycerols (Casparrini et al., 1968; Kino et al., 1982; Satouchi et al., 1978; Myher et al., 1978) and to the mass spectra of hydroxyoctadecadienoates (Hubbard et al., 1980). Interpretation of the ions at m/z 424, 353, and 324 (marked with an asterisk in Figure 4) was not so straightforward. By reference to the [$^2H_9$]Me_3Si spectrum (all three ions 9 amu higher) and to the hydrogenated spectrum (no equivalent to m/z 424, but the other two at 4 amu higher), the ion at m/z 424 was interpreted as $[CH_2CH(O_2R_2)CH{=}OH]^{\cdot+}$ and the related ions at m/z 353 and 324 as formed by losses of 71 (C_5H_{11}) and 100 (rearrangement with retention of Me_3Si and loss of the terminal six carbons of R_2 as hexenol), respectively. Loss of 100 amu is found in the Me_3Si derivatives of other hydroxy fatty acids [e.g., Maas et al. (1982)], and the fragmentation is discussed in Eglington et al. (1968).

A notable feature of the mass spectrum in Figure 4 is the absence of ions corresponding to loss of either of the acyl substituents as a M – ($RCOOCH_2$) fragment. This is diagnostic of a 1,2-diacylglycerol as opposed to the 1,3-diacyl analogue (Casparrini et al., 1968). It could be concluded that the structure of the diglyceride is 1-palmitoyl-2-(13-hydroxy-9-*cis*,11-*trans*-octadecadienoyl)glycerol. The very minor diglycerides observed on HPLC were presumed to represent 3% of the 9-hydroxy analogue, together with small amounts of the 13-hydroxy-9-*trans*,11-*trans* analogue plus some 1,3-diacyl analogue formed during the isolation procedure.

Although the diglyceride of Figure 4 chromatographed on GLC as a nice single peak, the 15-HETE analogue had weak shoulders with different ion abundances, and it was clear that it experienced some thermal degradation/isomerization. Shoulders were also present on the samples subjected to hydrogenation (PtO_2/H_2).

Stereospecificity of Oxygenation. Steric analysis of the configuration of the hydroxyl group was determined by HPLC. Figure 5 illustrates the predominantly *S* configuration of the hydroxylinoleate derived from the oxygenated phospholipid. Analysis of the 15-HETE from the phosphatidylcholine also gave a result of 97% *S* and 3% *R* enantiomer. In the case of the latter compound the designation of chirality was checked by oxidative ozonolysis of the menthoxycarbonyl derivative followed by GC–MS analysis of the reesterified 2-hydroxyheptanoate fragment (Hamberg, 1971). This completely independent method confirmed the assignment of stereochemistry.

Lipoxygenase Reaction in [^{18}O]*Water.* This was carried out to examine whether the polyunsaturated component of the

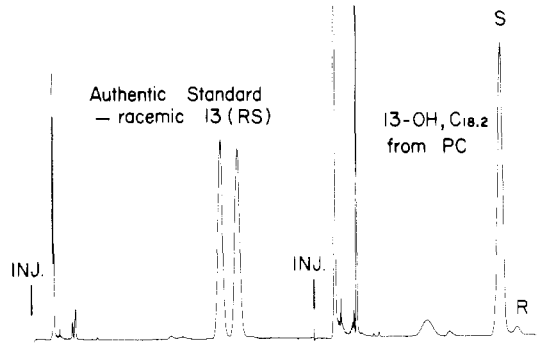

FIGURE 5: Stereospecificity of the oxygenation of phospholipid by soybean lipoxygenase. (Left side) Normal-phase HPLC of the diastereomers formed by reaction of *rac*-13-hydroxy-9(*Z*),11(*E*)-linoleate methyl ester with the isocyanate of dehydroabietylamine. (Right side) The corresponding derivative of 13-hydroxylinoleate recovered after reaction of 1-palmitoyl-2-linoleoylphosphatidylcholine with soybean lipoxygenase. Chromatographic conditions are given under Materials and Methods.

phosphatidylcholine remained coupled to the phospholipid during the oxygenation. If the ester bond were subject to hydrolysis in the presence of $H_2{}^{18}O$, the carboxyl group of the fatty acid would incorporate one atom of oxygen-18; if by some mechanism the fatty acid were re-esterified into the phospholipid, then 50% of the ^{18}O would be retained in the ester. The ^{18}O would then be stable throughout routine workup of the sample for GC–MS analysis. This was originally shown by Murphy and colleagues (Pickett & Murphy, 1981; Murphy & Clay, 1982), and subsequently, we checked each step in this transesterification, hydrolysis, HPLC, and derivatization procedure (Brash & Ingram, 1986).

Reaction of 1-palmitoyl-2-arachidonyl-PC with lipoxygenase was conducted in pH 9 borate diluted 50% with $H_2{}^{18}O$. Subsequently, the 15-HETE methyl ester isolated from the phospholipid product was hydrolyzed back to the acid and then converted to the pentafluorobenzyl ester trimethylsilyl ether derivative and analyzed by GC–MS (negative ion/chemical ionization). Selected ion monitoring of the carboxyl anion (*m/z* 391, formed by loss of the pentafluorobenzyl moiety), together with *m/z* 393, gave an ion ratio of *m/z* 393/391 of 0.097 in an unlabeled standard and 0.094 in the sample from the $H_2{}^{18}O$ incubation; this very slight difference is insignificant (and is in the wrong direction for ^{18}O incorporation during the reaction!). It could be concluded that the lipoxygenase reaction proceeded without any hydrolysis and reesterification of the acyl ester bond of the phospholipid.

Reaction of Soybean Lipoxygenase with Other Phospholipids. A limited number of suitable substrates were available. Nevertheless, it was possible to test 1-palmitoyl-2-[1-^{14}C]arachidonyl-PE and 1-stearoyl-2-[^{14}C]arachidonyl-PI in 50 μL sized reactions (400 μM substrate, pH 9, 10 mM deoxycholate, and 50 μg/mL enzyme for 1 h at room temperature) with analysis by direct injection onto HPLC. The RP-HPLC analysis showed that the PE and PI each gave a single more polar product with UV absorbance at 235 nm; (two-thirds of the PE and approximately one-third of the PI were converted—in comparison, PC was about 50% converted under the same conditions). On normal-phase HPLC the products chromatographed as slightly more polar than their respective phospholipid parent; this is evidence that the oxygenated product retains the PE or PI structure of the phospholipid substrate. After triphenylphosphine reduction and transesterification, the oxygenated fatty acid ester was identified as 15-HETE methyl ester by HPLC criteria (reversed phase and normal phase). An aliquot of each [1-^{14}C]-15-HETE methyl ester was mixed with 20 μg of the unlabeled racemic compound, and the absolute configuration was determined by HPLC of the carbamate diastereomers formed with the isocyanate of dehydroabietylamine. The radiolabel derived from the PE and PI products cochromatographed 96.5% and 96.3%, respectively, with the *S* enantiomer of the unlabeled 15-(*RS*)-HETE standard.

Discussion

These experiments establish that fatty acids esterified in phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol are acceptable substrates for soybean lipoxygenase 1. Reaction with a single molecular species of phospholipid gave a single product. The enzyme formed an ω6 hydroperoxide of the arachidonate or linoleate moiety of the phospholipid, and this hydroperoxide was almost exclusively of the Ls configuration. That the product was an intact phospholipid containing a HPETE was rigorously demonstrated by a number of chromatographic and spectroscopic criteria. Perhaps the one control experiment that should be highlighted was the enzyme incubation in ^{18}O-labeled water. There was no incorporation of ^{18}O into the product, showing that the fatty acid remained covalently bound to the phospholipid at all times throughout the procedure. The results of this experiment eliminate the possibility of a hydrolysis and reesterification cycle during the incubation with the soybean lipoxygenase. Clearly, the esterified polyunsaturated lipid was the substrate, and its HPETE analogue was the product. Until recently, only the rabbit reticulocyte lipoxygenase has been tested with ester substrates (Schewe et al., 1975), although the nature of the products was not examined. An independent study reported by Jong et al. (1985) has also tested the oxygenation of phospholipid substrate, in this case with a number of 15-lipoxygenases. Although the enzymatic product was not subjected directly to structural analysis, the findings from an analysis of the phospholipase A_2 hydrolyzed samples point to positional-specific oxygenation of the fatty acid esterified in phosphatidylcholine. These results are considerably extended in the present investigation to establish that esterified lipids can indeed be acceptable substrates for highly specific oxygenations.

Soybean lipoxygenase is the most thoroughly studied of the plant lipoxygenases, and many fundamental principles of lipoxygenase metabolism have been established with this enzyme as a model; in many respects it can be taken as an enzyme typical of this class of dioxygenase. The first comparable dioxygenase to be identified in animal tissue was the fatty acid cyclooxygenase. This enzyme was soon tested with phospholipids as substrates, and the results were negative (Lands & Samuelsson, 1967; Vonkeman & Van Dorp, 1967). Since that time, 12-, 5-, and 15-lipoxygenases have been discovered in animal tissues. The results of the present study indicate that a more thorough appraisal of the reactions with ester substrates is warranted.

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Ferric Uptake Regulation Protein Acts as a Repressor, Employing Iron(II) as a Cofactor To Bind the Operator of an Iron Transport Operon in *Escherichia coli*†

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ABSTRACT: The Fur (ferric uptake regulation) protein is a negative regulator of the aerobactin operon and of several other siderophore-mediated, high-affinity iron transport systems in *Escherichia coli*. The purified Fur protein and a plasmid containing a *lacZ* fusion to the aerobactin operon were used in conjunction with an in vitro coupled transcription/translation system to demonstrate that the Fur protein requires Fe(II) or certain other divalent metals as a cofactor to negatively regulate expression of the aerobactin operon. In a second set of experiments, using a restriction site protection assay, Fur was shown to bind to and block the aerobactin promoter in a metal-dependent fashion. It is concluded that Fur acts as a classical negative repressor that, under in vivo conditions, uses ionic Fe(II) as a corepressor. Our results support the hypothesis [Williams, R. J. P. (1982) *FEBS Lett. 140*, 3–10] that prokaryotic cells may contain a standing pool of free or loosely bound Fe(II) that is capable of acting in a regulatory capacity.

Iron is required for the growth of almost all living cells (Archibald, 1983). Among the significant roles of iron in biology are the transport and storage of oxygen, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides, and electron transport via disparate carriers spanning a redox potential of essentially 1 V. Although abundant, iron is largely unavailable in an aerobic environment at physiological pH owing to the vanishingly small value for the solubility product constant of the hydroxide. However, in contrast to the biologically beneficial effects just enumer-

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