Change in the Positional Specificity of Lipoxygenase 1 Due to Insertion of Fatty Acids into Phosphatidylcholine Deoxycholate Mixed Micelles†

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ABSTRACT: Linoleic and arachidonic acids were inserted into phosphatidylcholine deoxycholate mixed micelles (PDM-micelles) with their tail groups buried inside and carboxylic groups exposed outside. The fatty acid hydrophobic tail had a high affinity for the hydrophobic region of phosphatidylcholine micelles. The fatty acids inserted into phosphatidylcholine micelles were better substrates for soybean lipoxygenase 1 (LOX1) with two distinct pH optima at 7.0 and 10.0. With Tween 20-solubilized linoleic acid, the enzyme had a pH optimum at 9.0, exclusively forming 13-hydroperoxides. However, with linoleic and arachidonic acids inserted into PDM-micelles, LOX1 synthesized exclusively 9- and 5-hydroperoxides, respectively. The enzyme brought about the transformation of the substrate either at pH 7.4 or at 10.0, less efficiently at pH 10.0. However, the regioselectivity of the enzyme was not altered by increasing the pH from 7.4 to 10.0. Thus, LOX1 could utilize fatty acids bound to membranes as physiological substrates. The enzyme utilized the carboxylic group of linoleic and arachidonic acids inserted into the PDM-micelles as a recognition site to convert the compounds into 9- and 5-hydroperoxides, respectively. This was confirmed by activity measurements using methyl linoleate as the substrate. Circular dichroism measurement of LOX1 with PDM-micelles suggested that while there was a small change in the tertiary structure of LOX1, the secondary structure was unaffected. Soybean LOX1, which is arachidonate 15-LOX, acted as "5-LOX", thus making it possible to change the regiospecificity of the LOX1-catalyzed reaction by altering the physical state of the substrate.

Lipoxygenases are a family of non-heme iron-containing dioxygenases distributed widely throughout the plant and animal kingdoms. They catalyze the regio- and stereospecific oxygenation of polyunsaturated fatty acids, containing the (*Z*,*Z*)-1,4-pentadiene moiety, to furnish conjugated hydroperoxydiene fatty acids (*1*−*4*). These enzymes are designated as 5-, 12-, and 15-LOX[1] on the basis of their ability to oxygenate arachidonic acid at carbons 5, 12, and 15, respectively. Plant lipoxygenases catalyze the hydroperoxidation of linoleic acid as the first step in the biosynthesis of the growth regulatory molecule, jasmonic acid, and of factors involved in wound healing, traumatin and traumatic acid. Mammalian lipoxygenases catalyze the hydroperoxidation of arachidonic acid, initiating the synthesis of two families of potent physiological effectors such as leukotrienes and lipoxines (*5*, *6*). Many of the plant and animal LOXs possess dual or multiple positional specificities, synthesizing various isomers of fatty acid hydroperoxides. Soybean lipoxygenase 1 (LOX1) with unusual pH optima of 9−10 oxygenates linoleic and linolenic acids to 13(*S*)-HPODs, and with arachidonic acid, the oxidation occurs at carbon 15, giving 15(*S*)-HPOD. Several reports have shown that the specificity of lipoxygenase may be altered in vitro by varying the pH (*7*), aligning the methylene groups of the substrate at the active site as the major determinant of the reaction rate (*8*), changing the conformational state of the enzyme in different microenvironments (*9*), and binding of the enzyme to lipid storage organelles as in the case of cucumber lipid body lipoxygenase (*10*). Further, it has been reported that 12- and 15-LOXs from plants and animals are sensitive to the location of the reactive 1,4-pentadiene moiety relative to the methyl terminus, but not the carboxylate of fatty acid substrates (*8*, *11*). The activity of soybean LOX1 depends on the charge of the fatty acid substrate, reacting best with a charged substrate like linoleyl sulfate to form 13-HPOD (*12*, *13*). LOX1 has a higher affinity for a monomeric substrate than for a fatty acid incorporated in mixed micelles formed by fatty acid and detergents (*14*). The analysis of the mechanism of the lipoxygenase-catalyzed reaction is complicated because the substrate is not a simple soluble molecule but a long chain fatty acid that aggregates into a number of forms in solution, and its solubility differs with the type and concentration of the detergent and the ionic strength of the reaction medium. In this paper, the effect of insertion of fatty acids into PDM-micelles with the carboxylic group being directed toward aqueous outer space, leading to the consequent change in the positional specificity and pH optimum of LOX1, is reported.

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[1] Abbreviations: LOX, lipoxygenase; HPOD, hydroperoxide; PC, phosphatidylcholine; ANS, 1-anilino-8-naphthalenesulfonate; DOC, deoxycholic acid; PDM-micelles, phosphatidylcholine deoxycholate mixed micelles; FA, fatty acids.

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MATERIALS AND METHODS

Chemicals. Soybean LOX1 was isolated according to the method of Axelrod et al. (*15*), with some modifications as described previously (*16*). Potato tuber lipoxygenase was prepared and assayed as reported previously (*17*). Linoleic and arachidonic acids were from Nu Check prep. MN, methyl linoleate, and egg yolk phosphatidylcholine were from Sigma. ANS and sodium deoxycholic acid were purchased from Aldrich. The organic solvents were HPLC grade and were from Qualigens. Stock solutions of fatty acids were prepared with a concentration of 10 mM in 10% alcohol in the required buffer.

Preparation of the PDM-Micelles and Insertion of Fatty Acids into the Micelles. Mixed micelles were prepared using the mixture of phosphatidylcholine and deoxycholate. After solubilization of the PC and DOC in chloroform/methnol (2:1), the solvent was evaporated with a flash evaporator and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris-HCl (pH 7.4) and then sonicated for 5 min using a bath type sonicator. FA insertion into PDM-micelles was carried out by adding FA solubilized in ethyl alcohol to PDM-micelles with 50 mM Tris-HCl buffer (pH 7.4). The final concentration of ethyl alcohol in the final reaction mixture did not exceed 1.7 mM.

Electron Microscopy. PDM-micelle dispersions were fixed with a 2% solution of potassium phosphotungstate. A drop of the mixture was placed on a carbon-coated grid, and the excess solution was drained off. After drying, the grid was examined immediately in the electron microscope (JEM-1010, JEOL) operated at 60 kV.

Fluorescence Microscopy. PDM-micelles after the addition of ANS solution in 50 mM Tris-HCl buffer (pH 7.4) (PC:ANS molar ratio of 4) were immediately examined under a Leitz Diaplan fluorescence microscope (Wetzlar, Germany) fitted with exciter filter BP (376 nm) and a suppression filter block (470 nm). The magnification factor was 40×.

ANS Fluorescence Studies. Fluorescence measurements were performed with a Shimadzu RF 5000 spectrofluorophotometer. The temperature was maintained at 25 °C by circulating the water through the thermostated cuvette holder. The fluorescence titration of ANS and PDM-micelles was followed according to the method of Azzi (*18*). The sample was excited at 375 nm, and fluorescence emission was recorded at 476 nm. The interaction of fatty acid with PDM-micelles was followed by competitive ligand binding measurements according to the method of Aceto et al. (*19*). The PDM-micelles were saturated with ANS as reflected in the maximum relative fluorescence intensity, F_{max}, at 476 nm. To this PDM−ANS complex was gradually added FA, and the decrease in fluorescence, F, was recorded. The dissociation constant for the competing ligand fatty acid was determined using the equation $F_{max}/F = 1 + K_{ANS}/[ANS](1 + [FA]/K_d)$ (*19*), where K_{ANS} is the dissociation constant for PDM−ANS determined as described previously (*18*), [FA] is the concentration of FA, the competing ligand for ANS, added during the titration, and K_d is its dissociation constant. The reciprocal of the dissociation constant was used as equilibrium constant K_{eq}.

Assay of Lipoxygenase. Enzyme activity was assayed at pH 9.0 using 0.2 M sodium borate buffer containing 100 μM sodium linoleate dispersed in Tween 20 (23 μM) according to the method of Axelrod et al. (*15*). The increase in absorbance at 234 nm due to the appearance of conjugated diene hydroperoxide was followed using a 160A Shimadzu UV spectrophotometer and with a molar extinction coefficient of 25 000 M^{-1} cm^{-1}. One unit of enzyme was defined as the formation of 1 μmol of product per minute at 25 °C under standard assay conditions. For fatty acids inserted into PDM-micelles, activities were determined similarly by spectrophotometry. The formation of hydroperoxide was followed at 234 nm (ϵ = 25 000 M^{-1} cm^{-1}) in a solution of 50 mM Tris-HCl (pH 7.4) containing 100 μM micellar PC and 100 μM fatty acid solubilized in ethyl alcohol (the final concentration did not exceed 1.7 mM). The protein concentration was determined using an E_{280} of 14.0 (*15*).

Isolation and Characterization of Lipoxygenase Products. The products of the reaction of LOX1 with linoleic and arachidonic acids were isolated as described by Galliard and Phillips (*17*), and the products were identified by straight phase HPLC using a Shimadzu liquid chromatograph equipped with a Sorbax-sil column (4.6 mm × 250 mm, 5 μm particle size). Positional isomers of the methylated linoleic and arachidonic acid oxygenation products were separated by isocratic elution with the solvent system *n*-hexane/isopropyl alcohol/acetic acid (100:2:0.1 by volume) and a flow rate of 2 mL/min at 25 °C (*20*). The absorbance at 234 nm (conjugated dienes) was recorded. The products of potato lipoxygenase and soy LOX1 with Tween 20-solubilized fatty acids at pH 5.5 and 9.0 were used as standards (*3*, *7*, *21*).

Circular Dichroism Measurements. CD spectra were recorded with a Jasco J20C automatic recording spectropolarimeter at 25 °C. Dry nitrogen was purged before and during the course of the measurements. The slits were programmed to yield a 10 Å bandwidth at each wavelength. Near-UV CD was recorded with a 1 cm cell and far-UV CD with a 1 mm cell. The measurements were taken with 50 mM Tris-HCl (pH 7.4) both in the presence and in the absence of PDM-micelles.

RESULTS

The presence of both polar and nonpolar binding sites in mixed micelles is well-documented. Therefore, binding sites of the micelles can be utilized for insertion of amphiphiles such as fatty acids. To understand whether the polyunsaturated fatty acids inserted into PDM-micelles were substrates for LOX1, a systematic study was carried out for quantification of the affinity of fatty acids for PDM-micelles, and the results on the kinetic analysis of the oxidation of the inserted substrates and its effect on the regioselectivity and the pH optimum of the enzyme are presented in the following sections.

Preparation of PDM-Micelles and Their Characterization. To prepare an optically transparent solution of PDM-micelles, the ratio of DOC to PC was varied from 0.25 to 2.0. The phase transfer from a turbid to clear PC solution was observed at a DOC to PC molar ratio of 2.0. The electron micrograph picture is shown in Figure 1A, the dimensions of the micelles being 0.1 μm × 0.3 μm and the micelles being spherical. Since the average size of the mixed micelle depended on the relative and absolute amounts of surfactant and lipid that were present in the system (*22*), in the study presented here, all solutions had the same applied DOC to PC molar ratio of 2.0

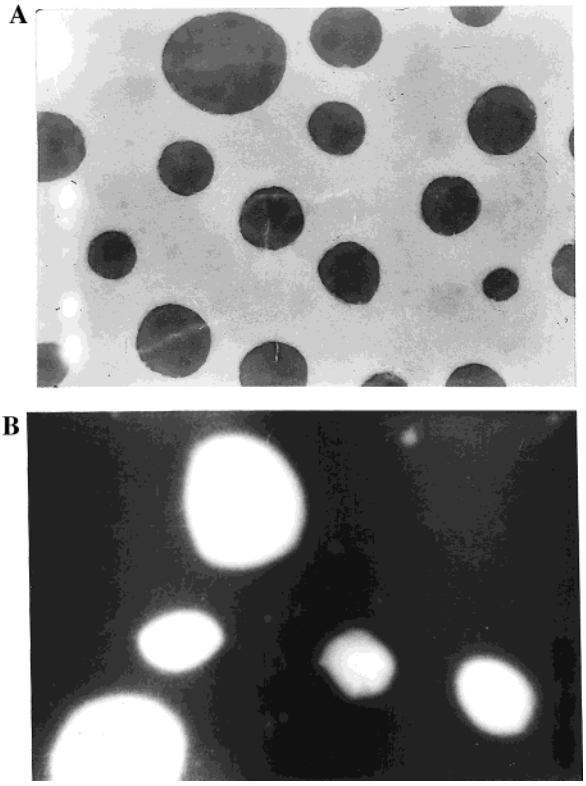

FIGURE 1: (A) Electron micrograph of PDM-micelles (DOC to PC molar ratio of 2.0) in 50 mM Tris-HCl (pH 7.4) fixed with a 2% solution of potassium phosphotungstate. Measurements were taken at 25 °C. (B) Fluorescence photomicrograph of the PDM-micelle−ANS complex in 50 mM Tris-HCl buffer (pH 7.4) (PC to ANS molar ratio of 4.0). The magnification factor was 40× [exciter filter BP (376 nm) and suppression filter (470 nm)].

Interaction of the Hydrophobic Fluorescent Probe ANS with PDM-Micelles. The interaction of the hydrophobic fluorescent probe ANS with PDM-micelles was followed by fluorescence microscopy. Immediately after the addition of ANS to PDM-micelles (PDM to ANS molar ratio of 4), luminous disks were observed as shown in Figure 1B, which suggested the binding of ANS to the hydrophobic pockets on the surface of PDM-micelles. To quantify further the binding of ANS to PDM-micelles, fluorescence titration measurements with ANS and PDM-micelles were taken. The ANS with PDM-micelles had an excitation maximum at 370 nm and an emission at 480 nm. The interaction of ANS with PDM-micelles was monitored by following changes in the relative fluorescence intensity of ANS as a function of its concentration. From the Scatchard plot of PDM−ANS interactions, it was inferred that one molecule of ANS was bound to four molecules of PC with an equilibrium constant of 0.4×10^4 M^{-1}. These data are in agreement with an earlier observation (*23*). The observations made in this study also suggested that ANS bound to the hydrophobic pocket formed by four PC molecules in mixed micelles.

Competitive Ligand Binding Measurements after Insertion of Fatty Acid into PDM-Micelles. With the addition of fatty acid to the PDM-micelle−ANS complex, the fluorescence intensity decreased with a concomitant red shift in the emission maxima of 18 nm (Figure 2). The interaction of fatty acid with PDM-micelles was quantified by competitive ligand binding measurements. These measurements with 50 mM Tris-HCl buffer (pH 7.4) indicated that the addition of fatty acid to the PDM-micelle−ANS complex, where fatty

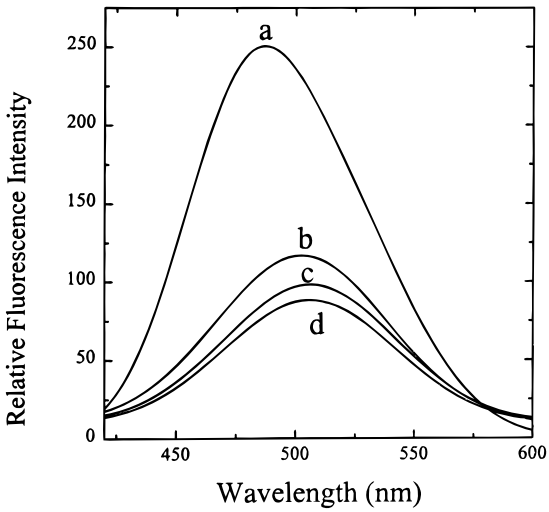

FIGURE 2: Fluorescence emission spectra of ANS in PDM-micelles and the effect of the insertion of fatty acid. Measurements were taken in 50 mM Tris-HCl buffer (pH 7.4) at 25 °C. The samples were excited at 375 nm; spectra were recorded for 3 mL of solution containing a micellar phosphatidylcholine concentration of 40 μM and ANS (10 μM) (a). To this solution was added 10 μM linoleic acid solubilized in ethyl alcohol, and the decrease in fluorescence intensity was observed (b). The corresponding blank spectra were recorded for a solution of 80 μM deoxycholic acid and 10 μM ANS (c) and 80 μM DOC and 10 μM ANS with 10 μM linoleic acid (d).

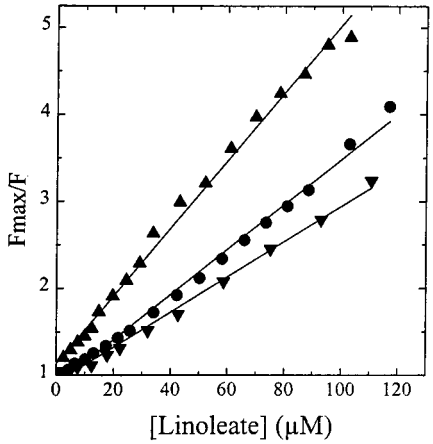

FIGURE 3: Competitive ligand binding measurements after insertion of linoleic acid into PDM-micelles. Fluorimetric titration of PDM-micelles and ANS with linoleic acid. Titration was performed at 25 °C by adding increasing amounts (5−100 μL) of a 2 mM stock solution of ANS to 2 mL of 50 μM PDM-micelles. After the saturation point (RFI_{max}), increasing amounts (5−100 μL) of 2 mM linoleic acid were added. The decrease in fluorescence intensity (RFI) was monitored; corrections were made for dilution, and the values were subtracted from the blank. Titrations were carried out in 50 mM Tris-HCl buffer (pH 7.4) (▲), in 50 mM sodium bicarbonate (pH 10.0) (●), and in 0.2 M borate buffer (pH 9.0) (▼).

acid replaced the ANS from PDM-micelles in a concentration-dependent manner, suggesting the insertion of fatty acid into the PDM-micelles. The equilibrium constant for the PC−fatty acid interaction was 1.3×10^4 M^{-1} (Figure 3). The addition of linoleic acid in 50 mM Tris-HCl buffer (pH 7.4) to DOC did not affect the fluorescence intensity of ANS in DOC (Figure 2, curves C and D), suggesting that fatty acid must be interacting with the PDM-micelles with its hydrophobic tail portion inserted into the micelles and carboxylic group protruding out.

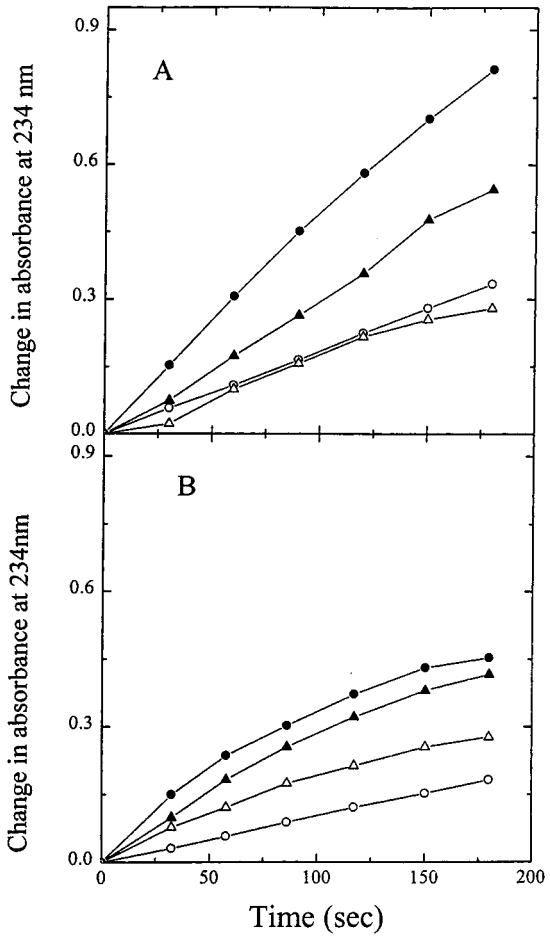

FIGURE 4: Oxygenation of fatty acids inserted into PDM-micelles or dispersed in Tween 20. The rates were measured by following the increase in absorbance at 234 nm at 25 °C. The concentration of LOX1 was kept the same for all the measurements. (A) Progress curve of LOX1-catalyzed oxidation of arachidonic acid: (i) 100 μM arachidonic acid inserted into 100 μM miceller PC in 50 mM Tris-HCl buffer (pH 7.4) (●) and in 16 mM sodium borate buffer (pH 9.0) (○) and (ii) 100 μM fatty acid in Tween 20 (23 μM) in 0.2 M sodium borate buffer (pH 9.0) (▲) and in 0.2 M sodium phosphate buffer (pH 7.4) (△). (B) Linoleic acid. The measurements were taken in the assay mixtures containing (i) 100 μM linoleic acid inserted into 100 μM miceller PC in 50 mM Tris-HCl buffer (pH 7.4) (●) and in 16 mM sodium borate buffer (pH 9.0) (○) and (ii) 100 μM fatty acid in Tween 20 (23 μM) in 0.2 M sodium borate buffer (pH 9.0) (▲) and in 0.2 M sodium phosphate buffer (pH 7.4) (△).

Oxygenation of Fatty Acid Inserted into PDM-Micelles. The rates of soybean LOX1-catalyzed oxidation of linoleic acid and arachidonic acid dispersed in Tween 20 and 0.2 M borate buffer (pH 9.0) and linoleic and arachdonic acids inserted into PDM-micelles with 50 mM Tris-HCl buffer (pH 7.4) were compared (Figure 4). The fatty acids inserted into PDM-micelles were better substrates at pH 7.4 than the Tween 20-solubilized fatty acid at pH 9.0. LOX1 activity decreased for Tween 20-solubilized fatty acid at neutral pH at high ionic strength, which was in agreement with an earlier observation (*14*). Although PC from egg yolk had 8% unsaturated fatty acids, being substrates for LOX1 (*24*), the PDM-micelles alone were not oxygenated by the enzyme and the LOX1 activity toward PDM-micelles alone was less than 1%.

Figure 5 shows the effect of the PC micellar concentration on the rate of dioxygenation of fatty acid at pH 7.4. The

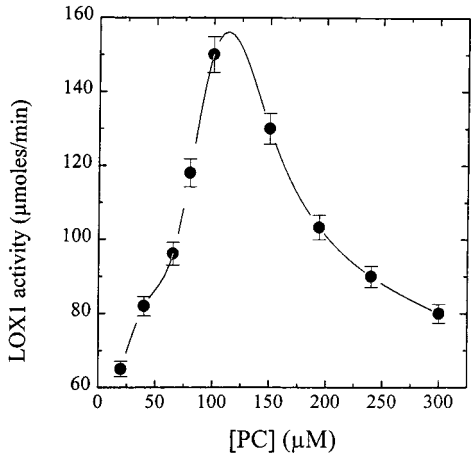

FIGURE 5: Effect of micellar PC concentration on the rate of LOX1-catalyzed linoleic acid oxidation. The enzyme assay was carried out in 50 mM Tris-HCl (pH 7.4) containing 100 μM linoleic acid, LOX1, and varying concentrations of micellar PC as PDM-micelles. The reaction was carried out for 3 min at 25 °C.

concentration of hydroperoxide that formed increased as the PC concentration increased to 100 μM. At a PC micellar concentration of 100 μM, the oxidation rate increased to 180 and 240% of that obtained in medium containing only deoxycholate and Tween 20, respectively. A further increase in the PC micellar concentration decreased the extent of oxidation. Thus, for the optimal fatty acid oxidation, the molar ratio of PC to fatty acid should be 1:1. The decrease in enzyme activity at high PC micellar concentrations can be explained in terms of a previous report (*14*). With the increase in the concentration of PC micelles, there was a 2-fold increase in the DOC concentration (DOC to PC molar ratio of 2). The presence of a large excess of DOC could affect the interaction of monomeric fatty acid with PDM-micelles by decreasing the effective free fatty acid concentration, thereby decreasing the reaction rate. It has also been suggested (*14*) that LOX1 had a higher affinity for monomeric substrate than for fatty acid incorporated in detergent micelles. However, the study presented here suggests that at neutral pH, the fatty acid inserted into PDM-micelles exhibited a relatively higher affinity for lipoxygenase.

The effect of increasing the substrate concentration on the activity of LOX1 under different conditions was studied. The reaction rates were found to follow Michaelis−Menten kinetics. The apparent values of K_m and V_{max} and the k_{cat}/K_m values are given in Table 1. The k_{cat}/K_m values obtained for fatty acid inserted into PDM-micelles at pH 7.4 clearly indicated that there was a 20% increase in the kinetic efficiency of the enzyme when compared to the activity at pH 9.0 toward Tween 20-dispersed substrates. The catalytic efficiency was higher for arachidonic acid than for linoleic acid. It has been shown that for LOX1, the k_{cat}/K_m for a given substrate is highly pH-dependent (*25*), and the enzyme becomes functionally inactive at low pH due to the large increase in K_m for Tween 20-solubilized substrate (Table 1). However, the K_m for fatty acid inserted into PDM-micelles with 50 mM Tris-HCl (pH 7.4) was comparable to that of the Tween 20-solubilized fatty acid at pH 9.0, suggesting that the enzyme could function equally well at either pH, provided the substrate was available in the right form to the enzyme for the catalysis.

Table 1: Kinetic Parameters for the Oxidation of Fatty Acid by Soybean LOX1 under Various Conditions[a]

substrate	pH	V_{max} (μmol min^{-1} mg^{-1} P)	k_{cat} (s^{-1})	K_m[b] (μM)	k_{cat}/K_m ($\times 10^7$ M^{-1} s^{-1})
LA[c] in Tween 20	7.4	80 ± 4	126	26 ± 1.6	0.48
	9.0	190 ± 9	298	12 ± 0.8	2.48
LA in PDM-micelles	7.4	226 ± 10	355	10 ± 0.7	3.55
	9.0	64 ± 4	101	29 ± 1.2	0.34
LA in PDM-micelles	10.0	210 ± 10	330	23 ± 1.6	1.43
AA[d] in Tween 20	7.4	162 ± 8	255	24 ± 1.8	1.06
	9.0	200 ± 11	314	11 ± 1.0	2.85
AA in PDM-micelles	7.4	260 ± 10	408	9 ± 0.4	4.53
	9.0	131 ± 7	205	28 ± 2.1	0.73

[a] Activity measurements taken with FA dispersed in Tween 20 were recorded at high ionic strength (0.6). Activity measurements taken with FA inserted into PDM-micelles were recorded at low ionic strength (0.05). [b] For comparative purposes, the concentration of FA represents the total concentration in the reaction mixture. [c] Linoleic acid. [d] Arachidonic acid.

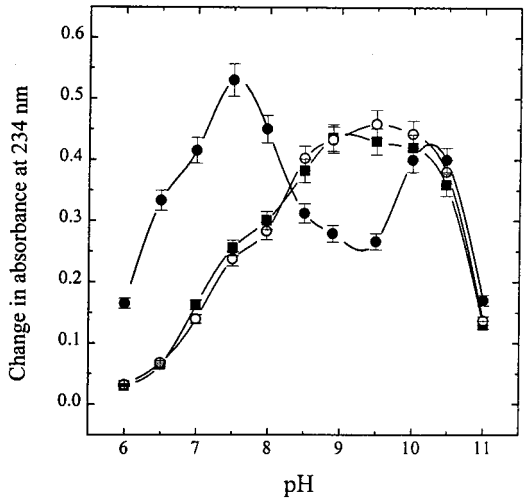

FIGURE 6: pH profile of LOX1 in the presence and absence of PDM-micelles. The enzyme was assayed in a series of buffers. The rate measured at each pH is expressed as the increase in absorbance at 234 nm at the end of 3 min. Enzyme activity measurements were taken with (i) 100 μM linoleic acid with 100 μM micellar PC at an ionic strength of 0.05 (●) and buffers 16 mM sodium phosphate (pH 6−7), 50 mM Tris-HCl (pH 7−8.5), 16 mM sodium borate (pH 8.5−9.5), and 25 mM sodium bicarbonate (pH 9.5−11), (ii) 100 μM linoleic acid in Tween 20 measured at an ionic strength of 0.6 (■) and buffers 0.2 M sodium phosphate (pH 6−8), 0.2 M sodium borate (pH 8−9.5), and 0.3 M sodium bicarbonate (pH 9.5−11), and (iii) 100 μM linoleic acid with 100 μM micellar PC at an ionic strength of 0.6 (○) and buffers 0.2 M sodium phosphate (pH 6−8), 0.2 M sodium borate (pH 8−9.5), and 0.3 M sodium bicarbonate (pH 9.5−11).

Effect of pH and Ionic Strength on the pH Activity Profile. The pH activity profile of the soybean enzyme as a function of pH and ionic strength is presented in Figure 6. The pH optimum for the Tween 20-dispersed linoleic acid at an ionic strength of 0.6 was in the alkaline range. However, the activity toward fatty acid inserted into PDM-micelles at a low ionic strength (0.05) exhibited optima around pH 7.4. Further, for PDM-micelles into which fatty acids had been inserted at low ionic strength (0.05), the presence of one more pH optimum around pH 10 was observed. With the fatty acid inserted into PDM-micelles at high ionic strength (0.6), the pH optimum shifted toward 9.0; either for Tween 20-dispersed linoleic acid at high ionic strength or with fatty acid inserted into PDM-micelles at high ionic strength, pH optima remained the same. Only at low ionic strength (0.05) with the fatty acid inserted into PDM-micelles were there two pH optima: one at pH 7.4 and another at pH 10.0. These changes could be due to the effect of pH and ionic strength on the insertion of FA into PDM-micelles.

The interaction of fatty acid with PDM-micelles was followed by competitive ligand binding measurements. The affinity of fatty acid for PDM-micelles at pH 10 and low ionic strength (0.05) decreased to 0.77×10^4 M^{-1} which could be due to the addition of negatively charged amphiphiles to negatively charged PDM-micelles limiting the amount of fatty acid that could be incorporated into PDM-micelles. These results, in turn, are reflected in a high K_m and a low k_{cat} for the enzyme at pH 10 (Table 1). At high ionic strength (0.6) and at pH 9.0, the equilibrium constant for FA with PDM-micelles decreased to 0.4×10^4 M^{-1} which could be due to the effect of high ionic strength on the hydrophobic interaction between FA and PDM-micelles.

Linoleic acid has an anomalous pK_a value of 7.9 (*13*), and the insertion of fatty acid into PDM-micelles could affect the pK_a value. Although fatty acids exist as either free fatty acids at very low concentrations or micelles at concentrations above the CMC, intermediate concentrations of fatty acids may lead to a substantial fraction of the lipid in dimeric forms or higher-order (premiceller) aggregates. It has also been shown that formation of the fatty acid−detergent complex occurs over a wide concentration range, and the subsequent ionization of acids within these complexes can occur at pH values well above 7 (*25*).

The competitive ligand binding measurements with PDM-micelles indicated that the fatty acids had a higher affinity for PDM-micelles than for the detergent. Therefore, with the addition of fatty acids to PDM-micelles (in a 1:1 molar ratio), fatty acid was inserted into PDM-micelles in relatively large amounts rather than with detergent micelles. An earlier report had suggested that the concentration of monomeric fatty acid was very low at neutral pH (*14*). Further, the report stated that the fatty acid molecule could interact with fatty acid binding sites on the detergent micelles also by a simple reversible equilibrium. These interactions reduced the effective monomeric fatty acid concentration at pH 7.4. Hence, PDM-micelles into which fatty acid had been inserted could be the substrate for LOX1.

Previously, it has been suggested that LOX1 had a greater affinity for charged substrates and a lower affinity for neutral ones, regardless of the pH of the solution (*24*), and interacted with monomeric substrate rather than with fatty acid inserted into detergent micelles. However, the results obtained in this study clearly show that LOX1 had a greater affinity for the fatty acid inserted into the PDM-micelles than for the monomeric substrate.

Regiospecificity of the LOX1-Catalyzed Reaction for the Micelle-Inserted Fatty Acid Substrate. The reaction products of LOX1 from linoleic and arachidonic acid inserted into PDM-micelles were identified by straight phase HPLC. The soybean LOX1 produced 13-HPOD and 15-HPOD from linoleic and arachidonic acid, respectively, solubilized with Tween 20 at pH 9.0 (ionic strength of 0.6). The potato LOX, using the same substrate, produced 9-HPOD and 5-HPOD at pH 5.5 from linoleic and arachidonic acid, respectively. The products of these two enzymes were used as authentic standards for the analysis of the product of LOX1 catalysis

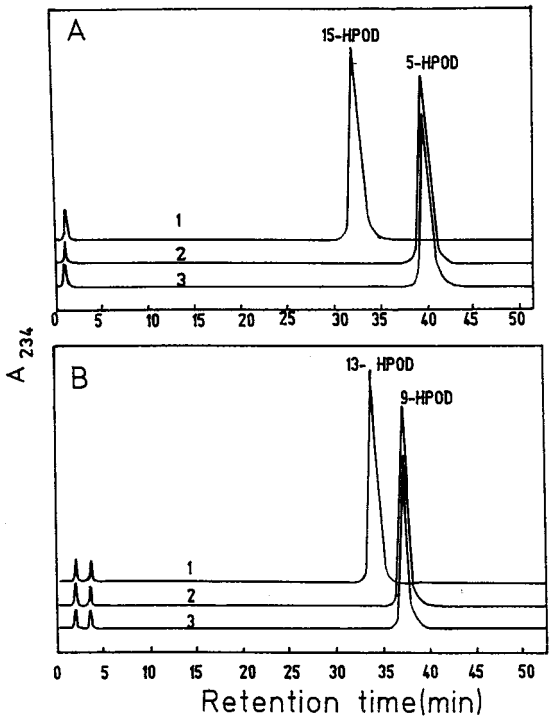

FIGURE 7: SP-HPLC analysis of fatty acid oxygenation products of lipoxygenase. The products of the reaction of LOX1 and potato lipoxygenase with arachidonic acid and linoleic acid as substrates were isolated, reduced, and methylated as described by Galliards and Phillips (*17*). The products were identified by SP-HPLC on a Sorbax-sil column (4.6 mm × 250 mm, 5 μm partical size). The products were separated by isocratic elution with the solvent system *n*-hexane/isopropyl alcohol/acetic acid (100:2:0.1 by volume) and a flow rate of 2 mL/min at 25 °C. The panels show the 234 nm profiles. The positions of the hydroxyl groups are denoted next to the individual peaks. (A) Oxygenation product of arachidonic acid. (1) Product of soybean LOX1 at pH 9.0 (0.2 M sodium borate buffer) using Tween 20-solubilized arachidonic acid as the substrate, (2) product of potato lipoxygenase at pH 5.5 with Tween 20-solubilized arachidonic acid as the substrate as standards (*21*), and (3) products of soybean LOX1 at pH 7.4 and 10.0 (25 mM sodium bicarbonate) using PDM-micelles into which arachidonic acid had been inserted as the substrate. (B) Oxygenation product of linoleic acid. (1) Product of soybean LOX1 at pH 9.0 (0.2 M sodium borate buffer) using Tween 20-solubilized linoleic acid as the substrate, (2) product of potato lipoxygenase at pH 5.5 with Tween 20-solubilized linoleic acid as the substrate as standards, and (3) products of soybean LOX1 at pH 7.4 and 10.0 (25 mM sodium bicarbonate) using PDM-micelles into which linoleic acid had been inserted as the substrate. The oxygenation products of fatty acid inserted into PDM-micelles formed by LOX1 at low ionic strength have the same retention times as the products formed by potato lipoxygenase with Tween 20-solubilized fatty acids.

using micelle-inserted fatty acid as the substrate (*3*, *7*, *21*). Figure 7 shows the product profile of LOX1-mediated oxidation products of linoleic and arachidonic acids. The retention time of the prominent peak obtained for the product of the LOX1 reaction with fatty acids inserted into PDM-micelles coincided with that of the peak obtained from the potato LOX. These results suggested that soybean LOX1 using fatty acid inserted into the PDM-micelles as the substrate produced exclusively 9-HPOD and 5-HPOD from linoleic and arachidonic acid, respectively. Previously, it has been shown that with the Tween 20-solubilized linoleic acid at pH 7.4, LOX1 produced almost equal proportions of 9-HPOD and 13-HPOD and at pH 7 a product composition 9-HPOD:13-HPOD ratio of 23:77 (*26*). However, this value did not change in the absence of Tween 20. Therefore, it was suggested that the regiochemical and stereochemical outcomes for the enzyme-catalyzed reaction were only slightly affected by the presence of Tween 20. However, the study presented here shows that the regiospecificity of LOX1 toward the linoleic acid inserted into the PDM-micelles was more specific in the synthesis of 9-HPOD at pH 7.4. Further, with the substrate, LOX1 exhibited one more pH optimum at 10.0 at low ionic strength (0.05) (Figure 6), while the product profile at this pH also exhibited the same profile that was obtained at pH 7.4, indicating the formation of 9- and 5-HPOD from linoleic and arachidonic acid, respectively. Although the enzyme exhibited a higher K_m and a low catalytic efficiency at pH 10.0 (Table 1), it was highly specific for 9-HPOD formation. However, at pH 10.0 and high ionic strength, LOX1 with Tween 20-dispersed substrates produced mainly 13- and 15-hydroperoxides from linolic and arachidonic acid, respectively. This type of complete change in regiospecificity toward the synthesis of only one regioiosmer would be possible only when the distal end (methyl end) of the fatty acid is not accessible to the LOX1. For fatty acids inserted into the micelles by their tail portion, the enzyme can approach fatty acid through its proximal (carboxyl group) end. The formation of 9-HPOD and 5-HPOD by LOX1 from linoleic and arachidonic acids with relatively low K_m and high k_{cat} values (Table 1) rules out the possibility of monomeric fatty acid as the substrate at pH 7.4. Similarly, at pH 10.0, although the concentration of monomeric fatty acid was high, the enzyme was oxygenating mostly fatty acid inserted into the PDM-micelles, but not the monomeric substrate. Since the fatty acid had a greater affinity for the PDM-micelles, a 2-fold excess detergent concentration (deoxycholic acid) can effectively reduce the monomeric form of fatty acid. With the Tween 20-solubilized substrate, both the rate of the reaction and regiospecificity changed due to the change in pH; a lower pH favors the formation of more 9-hydroperoxide. However, with fatty acids inserted into PDM-micelles at low ionic strength (0.05), only the rate of the reaction changed and not the regiospecificity. These results also supported the observation that the only PDM-micelle-inserted fatty acid is the substrate and not either the monomeric form or the detergent-mixed micellar fatty acid. Modification of the carboxylic group of linoleic acid led to a dramatic decrease in the reaction rate. Methyl linoleate solubilized in Tween 20 was shown to be a poor substrate at pH 9.0, as the LOX1 activity for this substrate was 11% of that of Tween 20-solubilized linoleic acid. The methyl linoleate inserted into the PDM-micelles was not dioxygenated at all the pH values that were studied. These results indicated that the carboxyl function of linoleic and arachidonic acids seemed to be essential for the dioxygenation by LOX1 when it was inserted into micelles.

Recently, it has been shown that the positional specificity of the cucumber lipid body lipoxygenase was altered after binding to the lipid storage organelle (*10*). Studies were carried out to determine whether the change in the positional specificity of LOX1 was due to binding of LOX1 to PDM-micelles. Polyacrylamide gel electrophoresis of LOX1 in the presence of PDM-micelles suggested that the LOX1 was moving as a single band with the same mobiltiy as its control (data not shown), which suggested that LOX1 was not binding to PDM-micelles. Circular dichroism measurements

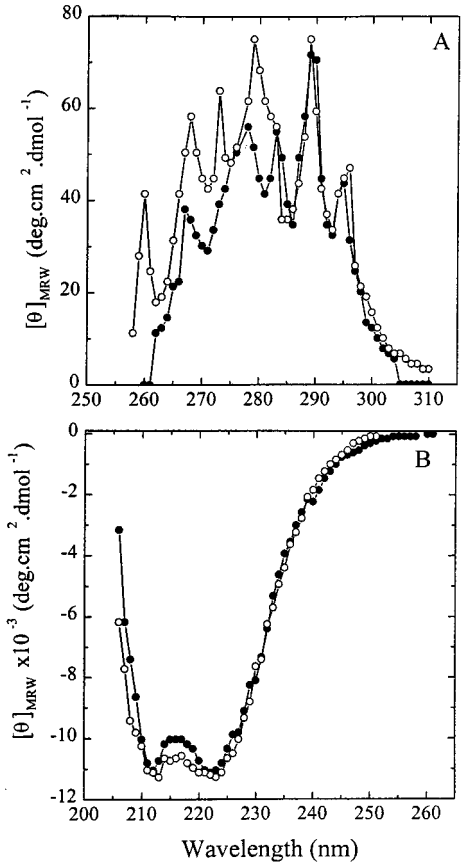

FIGURE 8: Circular dichroism spectra of LOX1 with PDM-micelles. (A) Near-UV CD. The protein concentration was 1 mg/mL in 50 mM Tris-HCl (pH 7.4), and the micellar PC concentration was 1 mM. (B) Far-UV CD. The protein concentration was 0.35 mg/mL with PC micellar concentrations of 0.5 mM: LOX1 (●) and LOX1 with PDM-micelles (○).

were taken for LOX1 both in the presence and in the absence of PDM-micelles. As shown in Figure 8, there were no significant changes in the secondary structure of LOX1 in the presence of micelles as evidenced by identical far-UV CD spectra. However, there were subtle changes in the tertiary structure as reflected by changes in the rotation in the phenylalanine absorption region.

DISCUSSION

The data generated from this study indicate that the unsaturated fatty acids inserted into PDM-micelles could serve as excellent substrates for soybean LOX1 catalysis. The most significant observation was the dramatic change in the pH activity profile toward the neutral pH with the subsequent change in regiospecificity in the synthesis of 9-HPOD and 5-HPOD from linoleic and arachidonic acid inserted into the PDM-micelles, respectively. The observed change in the regioselectivity of the enzyme and the shift in the pH optimum are due to the alterations in the physical state of the substrate. These observations have important implications in terms of membrane-associated polyunsaturated fatty acids as the physiological substrate of LOX1. Under physiological conditions, either polyunsaturated fatty acids exist mostly as aggregates or they are associated with the membrane (*25*). Most lipoxygenases exhibit a pH optimum around 7.0, and therefore, the fatty acids are no longer monomeric under these conditions. Thus, LOX1 can act on the fatty acids, which are also associated with the membranes.

An earlier report on the structure of micelles has shown the presence of hydrophobic pockets on the micelle (*23*). Fluorescence microscopy and competitive ligand binding studies characterized the binding of unsaturated fatty acids to such hydrophobic sites. Taken together, these results and the ability of LOX1 dioxygenation of fatty acid inserted into PDM-micelles indicated that only the small portion of the hydrophobic tail of the fatty acid could be interacting with the micelle in such a way that the pentadiene moiety remained exposed to the solvent. The exclusive formation of 5-HPOD and 9-HPOD from arachidonic and linoleic acid, respectively, by LOX1 suggested that the enzyme must have been approaching the bound substrate from its carboxy-terminal end. The substrates of lipoxygenases, arachidonic and linoleic acid, are held esterified at the second position of phospholipids of biological membranes. These unsaturated fatty acids are released through the action of specific phospholipase A2. Oxygenation of fatty acids by the lipoxygenase is thought to be regulated by the activation of phospholipase A2. Thus, the carboxyl end of the fatty acid generated by phospholipase A2 could be recognized by lipoxygenase for the oxygenation reaction.

Another interesting observation was that the k_{cat}/K_m value for the micelle-inserted substrate was higher. This could be due to the fact that the substrate was in a solution, which was better than the alcohol or Tween 20 dispersion, thus providing a sufficient population of substrate for a more productive encounter with the enzyme. Therefore, the method followed in this study could be adopted for the assay of lipoxygenases at neutral pH.

The product profile did not change at either pH optimum, viz., 7 and 10 for fatty acid inserted into PDM-micelles at low ionic strength. The lipoxygenases which act at neutral pH and produce 9-HPOD as the major product utilize the carboxylic acid group as a recognition site, rather than the ω-end of the fatty chain (*7*); that is, oxidation of several substrates occurs either at carbon 5 or 9 or not at all. These results are also in line with LOX1, which did not utilize the methyl linoleate inserted into the micelles. These observations clearly showed that LOX1 by utilizing the fatty acid inserted into the PDM-micelles could function as 5-LOX. The results of the studies presented here are further supported by the observation of Perez-Gilabert et al. (*27*). LOX1 oxidized dilinoleoylphosphatidylcholine at pH 7.5 and 10.0 (ionic strength of 0.6), resulting in the exclusive formation of 13-hydroperoxyoctadecadienoic acid derivatives of dilinoleoylphosphatidylcholine, which could be due to the blockage of the carboxylic end of the fatty acid.

The absolute change in the regiospecificity of LOX1 can have greater ramifications in the synthesis of physiological and/or pharmacological regulatory molecules derived from 5-HPODs. Further, each isomer of LOX1 products is a precursor of a different metabolic pathway (*28*−*30*). The results of this study demonstrate that a single enzyme can change its regiospecificity via changes in substrate presentation. Although the micellar system in these experiments may not reflect directly the event of lipid peroxidation occurring in vivo, observations made in this study may have physiological significance, involving lipoxygenase in biomembrane alteration, in both animals and plants.

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