Soybean Lipoxygenase-1 Oxygenates Synthetic Polyenoic Fatty Acids with an Altered Positional Specificity. Evidence for Inverse Substrate Alignment[†]

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ABSTRACT: The positional specificity is the decisive enzyme property for classification of lipoxygenases and for the currently used lipoxygenase nomenclature. It has been reported before that soybean lipoxygenase-1, which oxygenates polyenoic fatty acids at alkaline pH to the corresponding $n − 6$ hydroperoxy derivative, exhibits a different positional specificity when either the reaction conditions or the substrate structure is altered. To investigate the impact of structural substrate modifications on the positional specificity of this enzyme and to force an inverse substrate binding, we synthesized arachidonic acid analogues modified at the ω-terminus. Care was taken that the double bond system remained unchanged so that hydrogen abstraction from all three bisallylic methylenes was theoretically possible. We found that ω-modification of arachidonic acid leads to an impaired substrate affinity and a reduced reaction rate, but we did not detect any 5-lipoxygenation products, suggesting that structural modification of the ω-end may not be sufficient to force an inverse substrate orientation. However, when both ends of the fatty acid chain (ω-terminus and free carboxylate) were modified simultaneously, a considerable share of 5-lipoxygenation products was detected. These results indicate that introduction of polar or bulky groups at the methyl terminus of polyenoic fatty acids was not sufficient to force an inverse substrate orientation. However, simultaneous introduction of an ω-OH group and methylation of the carboxylate led to formation of significant 5-lipoxygenation products, suggesting an inverse head to tail substrate orientation.

Lipoxygenases (LOXs)[1] constitute a family of non-heme iron-containing dioxygenases distributed widely throughout the plant and animal kingdoms. They catalyze the regio- and stereospecific introduction of molecular dioxygen into polyunsaturated fatty acids to furnish conjugated diene-containing hydroperoxy fatty acids (*1*, *2*). Mammalian LOXs are classified with respect to their positional specificity of arachidonic oxygenation into 5-, 8-, 12-, and 15-LOXs (*3*, *4*). Since arachidonic acid is not a major polyenoic fatty acid in plants, plant physiologists prefer a linoleic acid-related enzyme nomenclature (*5*, *6*). Although a variety of LOX isoforms have been identified in plants and animals, the biological role of these enzymes is still a matter of discussion. In mammals, arachidonate 5-LOXs are key enzymes in the biosynthesis of leukotrienes, which are important mediators of inflammatory and anaphylactic diseases (*7*, *8*). Although several hypotheses exist for the biological role of other mammalian LOX isoforms, their relevance is far from clear. Plant LOXs have been implicated in the biosynthesis of phytohormones (*9*, *10*) and other signaling molecules involved, for instance, in wound healing (*11*, *12*). However, here again, the biological role of most LOX isoforms is still elusive.

The positional specificity of LOXs, which is decisive for enzyme classification, is not an absolute enzyme property but depends on the alignment of substrate fatty acids at the active site. At alkaline pH, soybean LOX-1 catalyzes the exclusive formation of $n − 6$ hydroperoxides regardless whether free fatty acid (*13*) or phospholipids (*14*, *15*) are used as the substrate. At neutral pH, however, the enzyme converts free linoleic acid to a mixture of (9*S*)- and (13*S*)-HPODE (*16*), whereas 13-lipoxygenation was dominant with dilinoleoylphosphatidylcholine (*15*). Recently, it has been reported that the positional specificity of soybean LOX-1 was altered severely when the substrate fatty acids were incorporated into phospholipid micelles (*17*). Under these conditions, the enzyme acted as arachidonate 5-LOX. In contrast, when the substrate was encapsulated into cyclodextrins, the reaction specificity was not influenced (*18*). At reduced oxygen pressure, soybean LOX-1 catalyzed both

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linoleic acid 9- and 13-lipoxygenation, and this product pattern was suggested to be due to leakage of radical intermediates from the active site of the enzyme (*19*).

The positional specificity of the LOX reaction depends on the substrate alignment at the active site (*20*). When soybean LOX-1 catalyzes an $n - 6$ oxygenation, the fatty acid substrate may slide into the substrate binding pocket (*21*−*23*) with its ω-end ahead so that the $n - 8$ bisallylic methylene is located in the proximity of the enzyme's hydrogen acceptor (*24*, *25*). When the chemical structure of the substrate is altered in such a way that $n - 8$ hydrogen abstraction is not possible any more [e.g., when 15-H(P)-ETE is used as the substrate], inversely bound substrate molecules may be oxygenated (*13*). A similar effect may result when the availability of the substrate ω-terminus is limited by incorporation of the fatty acid into micelles (*17*). These data suggest that the structural properties of LOX substrates as well as the manner of substrate presentation appear to be important for LOX specificity.

This study was designed to further explore the impact of structural alterations of substrate fatty acids on the reaction specificity of soybean LOX-1. For this purpose, we synthesized arachidonic acid analogues modified at either end of the hydrocarbon chain to force an inverse substrate alignment at the active site which should be reflected by the formation of a significant share of 5-lipoxygenation products.

MATERIALS AND METHODS

Chemicals. Commercial sources for the chemicals that were used were as follows: soybean LOX-1 (grade 1), arachidonic acid [(5Z,8Z,11Z,14Z)-eicosatetraenoic acid], dihomo-γ-linoleic acid [(8Z,11Z,14Z)-eicosatrienoic acid], ω-hydroxyarachidonic acid [20-hydroxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid], and sodium borohydride from Serva (Heidelberg, Germany); nitrosomethylurea and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma (Deisenhofen, Germany); 10% Pd/CaCO$_3$ (catalyst for hydrogenation) from Aldrich (Taufkirchen, Germany); and HPLC solvents from Merck (Darmstadt, Germany). The other ω-modified polyenoic fatty acids used in this study were synthesized as reported previously (*27*, *28*).

Assay System. The oxygenation kinetics of the different fatty acid derivatives were assayed spectrophotometrically by measuring the increase in absorbance at 235 nm in the substrate concentration range of 10−300 μM. The assay mixture consisted of 0.1 M sodium borate buffer (pH 9.0) containing various concentrations of substrate, and the reaction was started by addition of enzyme (5 μg). Before addition of the enzyme, the mixture was sonicated for 30 s to achieve homogeneous substrate dispersion. All measurements were carried out at room temperature.

For analysis of the oxygenation products, a 5 mL incubation was carried out under the same conditions as the kinetic measurements. After 5 min, the hydroperoxy fatty acids that formed were reduced to the corresponding hydroxy derivatives by the addition of a saturated ethanolic solution of sodium borohydride. After acidification to pH 3, the lipophilic products were twice extracted with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was reconstituted in 0.5 mL of methanol, and aliquots were injected into the RP-HPLC system.

Analytics. RP-HPLC was performed with a Shimadzu LC-6A liquid chromatograph connected to a Hewlett-Packard model 1040A diode array detector. Separation of the fatty acid derivatives was performed on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250 mm × 4 mm, 5 μm particle size) and a guard column (30 mm × 4 mm, 5 mm particle size, same vendor). For analysis of the hydroxylated fatty acids, solvent systems consisting of methanol, water, and acetic acid (80:20:0.1 or 70:30:0.1, by volume) and a flow rate of 1 mL/min were used. The absorbance at 235 nm was recorded. SP-HPLC was carried out on a Nucleosil 100-7 column (Macherey-Nagel; 250 mm × 4 mm, 5 μm particle size) with the *n*-hexane/2-propanol solvent system containing 0.1% acetic acid and a flow rate of 1 mL/min. The 2-propanol content varied between 2 and 10% depending on the chemical structure of the substrate.

For gas chromatography−mass spectrometry (GC−MS) experiments, the free hydroxy fatty acids were prepared by RP- and/or SP-HPLC and methylated with diazomethane and their hydroxy groups were silylated with bis(trimethylsilyl)trifluoroacetamide in dry pyridine. The GC−MS experiments were carried out on a Shimadzu GC-MS QP-2000 system equipped with a fused silica column SPB 1 (10 m × 0.25 mm, coating thickness of 0.25 μm). An injector temperature of 270 °C, an ion source temperature of 180 °C, and an electron energy of 70 eV were adjusted. The derivatized fatty acids were eluted with the following temperature program: from 130 to 200 °C at a rate of 50 °C/min and then to 260 °C at a rate of 5 °C/min, followed by a 10 min isothermal postrun.

Miscellaneous Methods. Protein concentrations were determined with the Roti-Quant kit (Carl Roth GmbH, Karlsruhe, Germany). For more informative mass spectra, the methylated derivatives of the polyenoic fatty acids (10 μg dissolved in 1 mL of ethanol) were hydrogenated using 5 mg of 10% Pd/CaCO$_3$ from Merck as a catalyst. Hydrogen gas was bubbled through this mixture for 2 min at room temperature. The solution was filtered to remove the catalyst; the solvent was evaporated, and the products were silylated as described above. After the removal of pyridine, the residue was reconstituted in 20 μL of dodecane and aliquots were injected into the GC−MS apparatus. Methylation of free carboxylates was achieved by bubbling etheric diazomethane with argon (2 min) and transferring the diazomethane gas to the fatty acid solution. For ethylation, etheric diazoethane (prepared from nitrosoethylurea according to the standard procedure described for diazomethane) was used. Monomethylation of the dicarboxylic fatty acid was achieved by treating the substrate with substoichiometric amounts of diazomethane. Then the monomethyl ester fraction was separated from the dimethyl esters and from the underivatized dicarboxylic acid by preparative RP-HPLC. Alkaline hydrolysis and complete methylation were carried out to check the chemical identity of the monomethyl ester.

RESULTS

Structural Alterations at the ω-End of Substrate Fatty Acids. Arachidonic acid (*13*, *25*) is oxygenated by soybean LOX-1 to (15S,5Z,8Z,11Z,13E)-15-hydroperoxy-5,8,11,13-eicosatetraenoic acid [(15S)-HPODE], and we confirmed these data in the study presented here. Since the distance of

Table 1: Structures and Nomenclature of LOX Substrates

free acid/methyl ester	R	substrate	structure
free acids	-CH$_2$-CH$_3$	18-CH$_2$-CH$_3$-C20:Δ5,8,11,14 (arachidonic acid)	
	-CH$_2$-J	18-CH$_2$-I-C19:Δ5,8,11,14	
	-CH$_2$-OH	18-CH$_2$-OH-C19:Δ5,8,11,14	
	-CH$_2$-CH$_2$-OH	18-CH$_2$-CH$_2$-OH-C2O:Δ8,11,14	
	-COOH	18-COOH-C19:Δ5,8,11,14	
	-CH$_2$-C(CH$_3$)$_3$	18-CH$_2$-C(CH$_3$)$_3$-C23:Δ5,8,11,14	
methyl esters	-COOH	18-COOH-C19:Δ5,8,11,14	
	-CH$_2$-OH	18-CH$_2$-OH-C19:Δ5,8,11,14	
	-CH$_2$-C(CH$_3$)$_3$	18-CH$_2$-C(CH$_3$)$_3$-C23:Δ5,8,11,14	

Table 2: Oxygenation of Arachidonate Derivatives Modified at the ω-Terminus[a]

R	substrate	V_{max} (s^{-1})	K_M (μM)
CH$_2$−CH$_3$	free acid	29.5	8.9
CH$_2$−I		21.1	194
CH$_2$−OH		2.7	39.4
CH$_2$−CH$_2$−OH		7.0	124
COOH		<0.1	nd
CH$_2$−C(CH$_3$)$_3$		<0.1	nd
CH$_2$−OH	methyl ester	4.8	18.4
COOH		4.2	4.3
CH$_2$−C(CH$_3$)$_3$		<0.1	nd

[a] The various arachidonate derivatives were oxygenated by soybean LOX-1 (5 μg/mL) at pH 9.0 in the standard assay system (Materials and Methods). Apparent K_M and V_{max} values were extracted from Lineweaver−Burk plots (measurements at five different substrate concentrations).

the bisallylic C$_{13}$ (site of initial hydrogen abstraction) from the ω-end of the substrate is important for both reaction rate and positional specificity (*24, 25*), we altered this distance by introducing different ligands at the substrate's ω-position (Table 1). First, we replaced the ω-terminal CH$_3$ group with a bulky iodine (18-CH$_2$-I-C19:Δ5,8,11,14; Table 1). This modification was supposed to increase the critical distance and, thus, may dislocate the bisallylic C$_{13}$ from the enzyme's hydrogen acceptor. Soybean LOX-1 was capable of oxygenating this artificial substrate, suggesting that the ω-iodine prevents neither substrate binding nor the introduction of dioxygen. However, the reaction kinetics were quiet different from those of arachidonic acid oxygenation. The high K_M indicates a lower binding affinity, and V_{max} was strongly reduced (Table 2). RP-HPLC analysis of the reaction products indicated one major conjugated diene (Figure 1), which was shown to be homogeneous via SP-HPLC (data
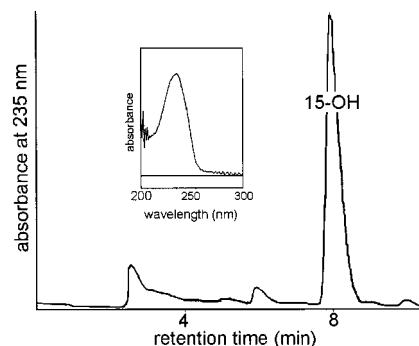

FIGURE 1: RP-HPLC analysis of the reaction products formed from 18-CH$_2$−I−C19:Δ5,8,11,14 by soybean LOX-1. Soybean LOX-1 (5 μg) was incubated with 200 μM 18-CH$_2$−I−C19:Δ5,8,11,14 at room temperature in 1 mL of 0.1 M borate buffer (pH 9.0). After the reaction was completed (no further increase in absorbance at 235 nm), the hydroperoxy lipids that formed were reduced to the corresponding hydroxy compounds by the addition of a molar excess of sodium borohydride. The mixture was acidified to pH 3, and the lipophilic products were extracted twice with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was reconstituted in 0.5 mL of methanol, and aliquots were injected into the RP-HPLC apparatus with the methanol/water/acetic acid solvent system (80:20:0.1, by volume). The absorbance at 235 nm was monitored. The inset shows the UV spectrum of the major oxygenation product indicating a conjugated diene chromophore.

not shown). Its electron impact mass spectrum exhibited a major ion at m/z 337 [base peak; CH=CH−CH=CH−COTMS−(CH$_2$)$_4$−I] and a minor one (4%) at 518 (M$^+$). The spectrum of the hydrogenated derivative was characterized by the α-cleavage ions at m/z 343 (25%) and 159 (100%) as well as by the M$^+$ − 15 ion at m/z 382 (1%). If one considers that hydrogenation reduced the ω-terminal CH$_2$I group to CH$_3$, these data indicate that the ω-iodine substrate was oxygenated exclusively at C$_{15}$.

Next we synthesized an arachidonic acid analogue, in which an OH replaced the ω-CH$_3$. In this substrate (18-CH$_2$−OH−C19:Δ5,8,11,14; Table 1), the distance between the ω-terminus and the bisallylic C$_{13}$ is by ∼0.2 Å shorter than in arachidonic acid. As a consequence of that, a lower reaction rate and a dual positional specificity were predicted. Moreover, the presence of the polar ω-OH was supposed to impair substrate binding because the ω-end of the fatty acid has to be buried in the hydrophobic substrate binding pocket. Our kinetic data indicate that, compared with arachidonic acid, this substrate binds 4 times less efficiently at the active site and its V_{max} was by 1 order of magnitude lower (Table 2), and these data are in line with our prediction. Analyzing the oxygenation products in RP-HPLC, we observed two major conjugated dienes (Figure 2), which were shown to be homogeneous via SP-HPLC (not shown). The mass spectra of the TMS derivatives and of the hydrogenated derivatives (Table 3) suggested a major 15-lipoxygenation. The early-eluting minor product (Figure 3) was identified as a 12-hydroxy derivative. Thus, as predicted, soybean LOX-1 exhibits a dual positional specificity with this particular substrate. It is of mechanistic interest that we observed a singular positional specificity (exclusive 15-lipoxygenation) when ω-hydroxyarachidonic acid (18-CH$_2$−CH$_2$−OH−C20:Δ5,8,11,14) or the corresponding ω-hydroxy-dihomo-γ-linolenic acid (18-CH$_2$−CH$_2$−OH−C20:Δ8,11,14) was used as a substrate (data not shown). Since the
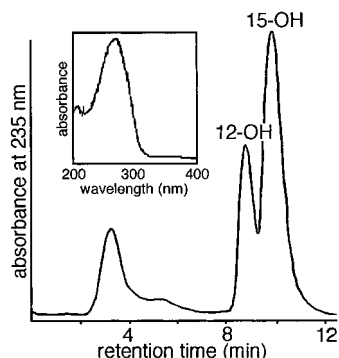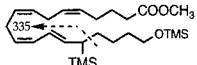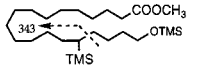
FIGURE 2: RP-HPLC analysis of the reaction products formed from 18-CH$_2$−OH−C19:Δ5,8,11,14 by soybean LOX-1. Soybean LOX-1 (5 μg) was incubated with 200 μM 18-CH$_2$−OH−C19:Δ5,8,11,14 at room temperature in 1 mL of 0.1 M borate buffer (pH 9.0). After the reaction was completed (no further increase in absorbance at 235 nm), the hydroperoxy lipids that formed were reduced to the corresponding hydroxy compounds by the addition of a molar excess of sodium borohydride (saturated ethanolic solution). The mixture was acidified to pH 3, and the lipophilic products were extracted twice with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was methylated with diazomethane (see Materials and Methods), and the methyl esters were reconstituted in 0.5 mL of methanol. Aliquots of this solution were injected into the RP-HPLC apparatus with the methanol/water/acetic acid solvent system (70:30:0.1, by volume). The absorbance at 235 nm was monitored.

Table 3: GC−MS Data Obtained for the Late-Eluting (15-OH) Conjugated Diene Formed from 18-CH$_2$−OH−C19:Δ5,8,11,14[a]

[a] The late-eluting conjugated diene was prepared from 18-CH$_2$−OH−C19:Δ5,8,11,14 as described in the legend of Figure 2. After methylation of the free carboxylate and silylation of the OH, GC−MS was carried out as described in Materials and Methods.

methyl ends of these fatty acids are by one CH$_2$ unit longer than that of 18-CH$_2$−OH−C19:Δ5,8,11,14 (Table 1), these data will fit into the picture.

As a major reason for the low binding affinity of ω-hydroxylated fatty acids, the polarity of the ω-substituent may be discussed. To substantiate this hypothesis, we oxidized the ω-OH to the corresponding carboxylate. This derivatization led to a symmetric dicarboxylic fatty acid (18-COOH−C19:Δ5,8,11,14; Table 1), which from the geometric point of view should be a suitable substrate for soybean LOX-1 if the enzyme can tolerate a free carboxylate in the substrate binding pocket. Unfortunately, we found that this dicarboxylic fatty acid was not oxygenated, suggesting that
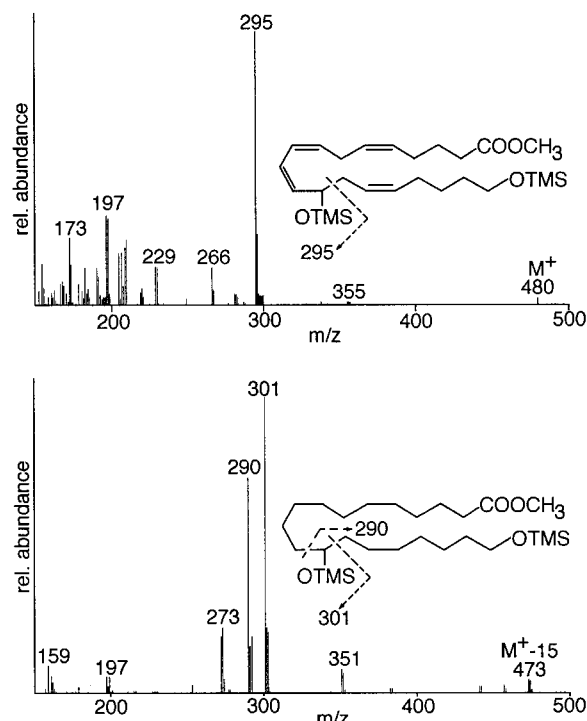

FIGURE 3: Mass spectra obtained for the minor oxygenation product formed from 18-CH$_2$−OH−C19:Δ5,8,11,14 by soybean LOX-1. Incubation of soybean LOX-1 (5 μg) with 200 μM 18-CH$_2$−OH−C19:Δ5,8,11,14 and RP-HPLC purification of the reaction products as methyl esters were carried out as described in the legend of Figure 2. The conjugated dienes that eluted at 9.7 (major product) and 8.7 min (minor product) were collected, and the solvent was evaporated under vacuum. The OH groups were derivatized (see Materials and Methods), and the products were analyzed by GC−MS. To obtain more informative mass spectra, the compounds were hydrogenated before derivatization. Mass spectra and fragmentation patterns are shown for the minor oxygenation product. The mass spectral data for the major oxygenation product are summarized in the Table 3. Both products were reanalyzed via SP-HPLC with the *n*-hexane/2-propanol/acetic acid solvent system (100:6:0.1, by volume) and found to be homogeneous when the absorbance at 235 nm was recorded.

the additional carboxylate group prevents the LOX reaction.

Next, we synthesized a LOX substrate in which the ω-CH$_3$ group of arachidonic acid was replaced with a bulky hydrophobic C(CH$_3$)$_3$ group. Since we did not observe (Table 2) any sizable oxygenation of this substrate (18-CH$_2$−C(CH$_3$)$_3$−C23:Δ5,8,11,14; Table 1), one has to conclude that the enzyme may not tolerate such a bulky residue at the ω-terminus of the substrate. As a mechanistic reason for this behavior, one may discuss the fact that the bisallylic C$_{13}$ is more distant from the ω-terminus as in arachidonic acid, which supposedly leads to suboptimal substrate alignment (displacement of the prochiral center from the enzyme's hydrogen acceptor).

Simultaneous Derivatization of the ω-Terminus and the Carboxylate Group. As a major reason for the impaired substrate behavior of ω-carboxylated fatty acids, one may assume that the polar ω-residues can hardly be buried in the hydrophobic environment of the substrate binding pocket because of energetic constraints. To obtain experimental evidence for this assumption, we methylated one carboxylate of the dicarboxylic substrate and tested the monomethyl ester (18-COOH−C19:Δ5,8,11,14 monomethyl ester; Table 1) as a substrate for the LOX-1. Surprisingly, we found (Table 2)
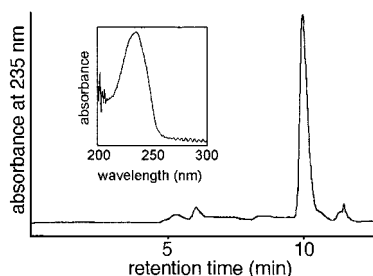
FIGURE 4: HPLC analysis of the reaction products formed from 18-COOH−C19:Δ5,8,11,14 monomethyl ester by soybean LOX-1. Soybean LOX-1 (5 μg) was incubated with 200 μM 18-COOH−C19:Δ5,8,11,14 monomethyl ester at room temperature in 1 mL of 0.1 M borate buffer (pH 9.0). After the reaction was completed (no further increase in absorbance at 235 nm), the hydroperoxy lipids that formed were reduced to the corresponding hydroxy compounds by the addition of a molar excess of sodium borohydride (saturated ethanolic solution). The mixture was acidified to pH 3, and the lipophilic products were twice extracted with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was reconstituted in 0.5 mL of methanol, and aliquots were injected into the RP-HPLC apparatus with the methanol/water/acetic acid solvent system (80:20:0.1, by volume) (data not shown). The only conjugated diene was collected, ethylated (see Materials and Methods), and reanalyzed via SP-HPLC with the *n*-hexane/2-propanol/acetic acid solvent system (100:5:0.1, by volume). The inset shows the UV spectrum of the major conjugated diene.

that this monomethyl ester was oxygenated with a fairly high reaction rate, although the binding affinity was impaired (compared with that of arachidonic acid). Since the dicarboxylic fatty acid that was synthesized was a symmetric molecule, it was of particular interest to determine whether the substrate was oxygenated close to the methylated or the free carboxylate and whether the enzyme exhibited a singular or dual positional specificity. RP-HPLC of the oxygenation products indicated one major conjugated diene (Figure 4) which was identified as a product of 15-lipoxygenation (free carboxylate defined as C_1). These data indicate that oxygen was introduced close to the methylated carboxylate, suggesting that the substrate may slide into the substrate binding pocket with the methyl ester group ahead.

It is shown in Figure 2 that soybean LOX oxygenated the *ω*-hydroxylated substrate 18-CH$_2$−OH−C19:Δ5,8,11,14 (Table 1) to a mixture of 12- and 15-hydroperoxy derivatives. These data suggest that the OH group can be buried in the hydrophobic environment of the substrate binding pocket despite the energetic constraints countering this process. Such constraints that have been reported previously for another *ω*-hydroxy fatty acid (*27*) may be reflected in the high K_M, indicating a strongly reduced binding affinity. Methylation of the free carboxylate increases the binding affinity and doubles the reaction rate (Table 2). These data were rather surprising since methylation of both arachidonic acid and linoleic acid dramatically impaired the substrate behavior (data not shown). RP-HPLC analysis (Figure 5A) of the reaction products of the methylated *ω*-hydroxy fatty acid revealed the formation of two conjugated dienes (products I and II). Via SP-HPLC (Figure 5B), the early-eluting minor product (I) was homogeneous (not shown), whereas the late-eluting compound (II) was split into two conjugated dienes (IIa and IIb). Products I and IIb were identified by GC−MS as 12- and 15-hydroxylated substrates, and their mass spectra were very similar to those shown in Figure 3 and Table 3. The spectra for compound IIa (Figure
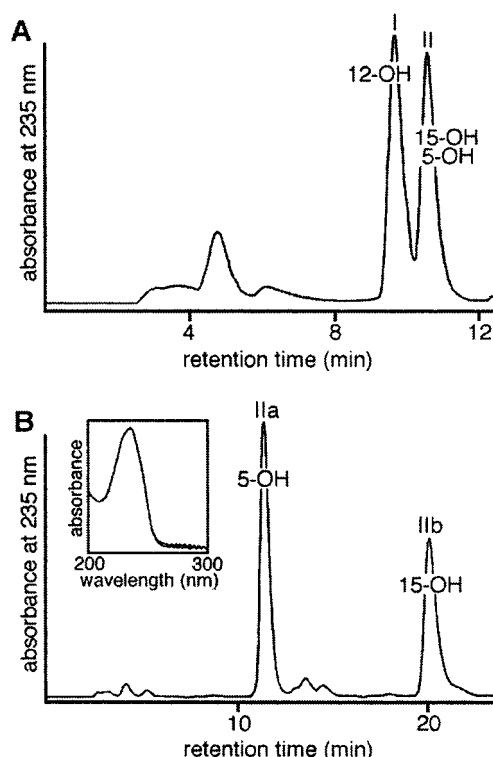

FIGURE 5: HPLC analysis of the reaction products formed from 18-CH$_2$−OH−C19:Δ5,8,11,14 methyl ester by soybean LOX-1. Soybean LOX-1 (5 μg) was incubated with the monomethyl ester of 18-CH$_2$−OH−C19:Δ5,8,11,14 (200 μM final concentration) at room temperature in 1 mL of 0.1 M borate buffer (pH 9.0). After the reaction was completed (no further increase in absorbance at 235 nm), the hydroperoxy lipids that formed were reduced to the corresponding hydroxy compounds by the addition of a molar excess of sodium borohydride (saturated ethanolic solution). The mixture was acidified to pH 3, and the lipophilic products were extracted twice with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was reconstituted in 0.5 mL of methanol, and aliquots were injected into the RP-HPLC apparatus (A) with the methanol/water/acetic acid solvent system (70:30:0.1, by volume). The absorbance at 235 nm was monitored. SP-HPLC (B) of the late-eluting conjugated diene was carried out with the *n*-hexane/2-propanol/acetic acid solvent system (100:6:0.1, by volume). The inset shows the UV spectrum of oxygenation products BI and BII, which represent a conjugated diene chromophore.

6) clearly indicate 5-lipoxygenation. Summarizing the product pattern formed from the methyl ester of the *ω*-hydroxylated substrate (18-CH$_2$−OH−C19:Δ5,8,11,14; Table 1), one may conclude that 12-lipoxygenation is the major reaction (46%) that is catalyzed, but more than one-third of the reaction products originate from 5-lipoxygenation (37%). Only ∼17% of the oxygenation products are formed via 15-lipoxygenation. These data suggest that a significant share of inversely bound substrate was oxygenated by the enzyme.

The free fatty acid of the *ω*-branched substrate [18-CH$_2$−C(CH$_3$)$_3$−C23:Δ5,8,11,14; Table 1] was not oxygenated by soybean LOX-1, and steric constraints have been discussed as possible reasons (see above). Unfortunately, the corresponding methyl ester was not a substrate for the enzyme (Table 2).

DISCUSSION

The positional specificity of LOXs, which is the basis for the currently used LOX nomenclature, is not an absolute
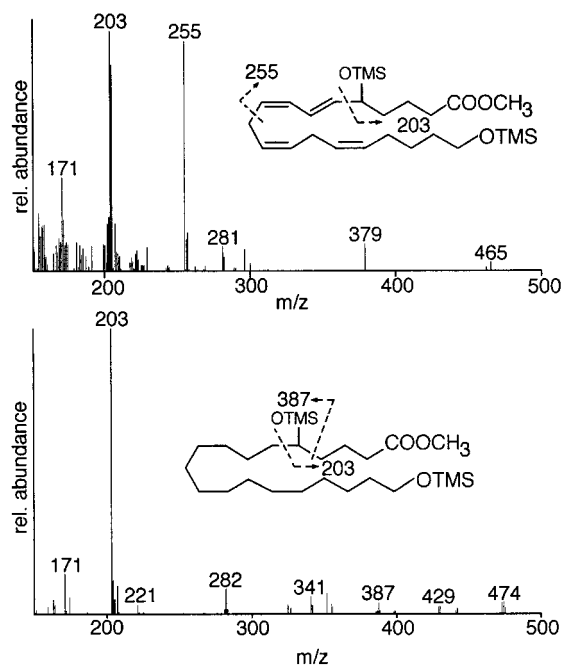
FIGURE 6: Mass spectrum obtained for the 5-lipoxygenation product formed from $18\text{-}CH_2\text{-}OH\text{-}C19:\Delta5,8,11,14$ methyl ester by soybean LOX-1. Soybean LOX-1 (5 μg) was incubated with the methyl ester of $18\text{-}CH_2\text{-}OH\text{-}C19:\Delta5,8,11,14$ (200 μM final concentration) at room temperature in 1 mL of 0.1 M borate buffer (pH 9.0). The late-eluting conjugated diene (10.5 min) was prepared by RP-HPLC (see the legend of Figure 5), and the two oxygenation products, which coelute on RP-HPLC, were separated by SP-HPLC. The late-eluting product (IIb) was identified as the 15-OH derivative by co-injection with an authentic standard, and the early-eluting diene was prepared for GC–MS (see Materials and Methods).

enzyme property but depends on substrate structure (*13, 24, 25*), on reaction conditions (*16, 19*), and on the physico-chemical state of the substrate (*17*). It has been suggested that substrate orientation at the active site is important for the specificity of the LOX reaction (*16, 20, 24, 25*). Soybean LOX-1, which exhibits a singular positional specificity with arachidonic acid [exclusive 15-H(P)ETE formation], oxygenates (15*S*)-HPETE to (8*S*,15*S*)- and (5*S*,15*S*)-DiH(P)ETE (*13*). Thus, it exhibits a dual positional specificity with this substrate. The stereochemistry of (15*S*)-HPETE oxygenation is consistent with an inverse substrate orientation at the active site. In this case, the fatty acid was supposed to slide into the substrate binding cleft with its carboxylate ahead. The principle possibility of such an inverse substrate orientation was first realized in mechanistic studies on the stereoselectivity of hydrogen abstraction by LOXs from corn and soybeans (*27*). Later, an inverse lipid alignment was suggested as a mechanistic reason for the oxygenation of linoleaidic acid by soybean LOX-1 (*30*), for the 9*S*-oxygenation of linoleic acid at neutral pH by the same enzyme (*16*)*,* and for the oxygenation of (15*S*)-HETE derivatives by the rabbit reticulocyte 15-LOX (*31*). In all of these experiments, substrate fatty acids were used that either contained a single bisallylic methylene (no choice of hydrogen abstraction for the enzyme) or lacked the $n-8$ prochiral center ($n-6$ lipoxygenation not possible). Our data indicate for the first time that 15-LOXs are capable of catalyzing major 5-lipoxygenation (hydrogen abstraction from C-7) even though 15-lipoxygenation is theoretically possible. It should be stressed at this point that there are

reports in the literature that emphasize a conserved substrate orientation for all LOX isoforms. The major argument against an inverse substrate alignment is the energy penalty associated with burying the polar carboxylate in the hydrophobic environment of the substrate binding pocket (*32, 33*). For certain LOX isoforms, such as soybean LOX-3, this energy barrier appears to be reduced because the carboxylate may interact with Arg726, which appears to be exposed at the surface of the substrate binding pocket (*23*). For other LOX isoforms, such as the human 5- and 15-LOXs, no polar or even charged side chain residues are present at this position or elsewhere at the bottom of the substrate binding cleft (*32*). However, if the substrate is inversely aligned, the energy barrier should be mirrored by an impaired substrate binding affinity. In fact, the affinity constant (K_M) for the binding of $18\text{-}CH_2\text{-}OH\text{-}C19:\Delta5,8,11,14$ was 4-fold higher than for arachidonic acid (Table 2), and previous investigations on 5-lipoxygenation of 15-H(P)ETE by the LOXs from soybeans (isoenzyme 1) and rabbits also revealed higher K_M values (*13, 31*).

Our findings and the data obtained from the literature suggest the ability of soybean LOX-1 to tolerate both the methyl end and the carboxyl terminus of fatty acid substrates in the substrate binding pocket despite considerable thermodynamic constraints. Recently, crystallographic studies have been carried out on the soybean LOX-3–13-HPODE complex (purple enzyme), and the data that were obtained indicate an "inverse" orientation of the probe. The electron density map clearly indicated that the free carboxylate is localized deep inside the substrate binding pocket (*34*).

With a defined substrate, there appears to be a binding equilibrium between straight and inverse substrate alignment, and this steady state may be influenced by the functional groups on either end of the hydrocarbon chain. With polyenoic fatty acids, the methyl terminus slides into the binding pocket. Introduction of polar or bulky ω-residues may shift the equilibrium toward an inverse orientation. However, for ω-hydroxylated fatty acids, this shift may be not be strong enough to force a sizable oxygenation of an inversely bond substrate; we still observed a major $\omega-6$ oxygenation. If in addition the polar carboxylate is methylated, the shift in binding equilibrium is likely to be more pronounced, because the polarity of the carboxylate is reduced and, thus, the energy barrier associated with burying this part of the substrate molecule in the hydrophobic environment of the active site may be much smaller. Our data, which indicate that a considerable share (37%) of $18\text{-}CH_2\text{-}OH\text{-}C19:\Delta5,8,11,14$ methyl ester is oxygenated at C_5 of the hydrocarbon chain, support this hypothesis. It is important to note that the lack of 5-lipoxygenation products does not rule out the possibility of an inverse substrate binding. It may be possible that the hydrogen removal from the inversely aligned substrate is sterically hindered, for instance, by a large distance of the doubly allylic methylene from the enzyme's hydrogen acceptor. In such a case, a considerable share of the substrate may be bound inversely, but this may not be reflected by the pattern of the oxygenation products. Thus, abortive substrate binding may interfere with the oxygenation process. Direct proof for an inverse substrate orientation can only be provided by crystallization studies of enzyme–substrate complexes. However, such crystal trials are difficult to perform since

they have to be carried out under strictly anaerobic conditions. Such conditions can hardly be reached under atmospheric conditions, and crystal trials in orbit are rather expensive.

Although our data suggest that 5-lipoxygenation of arachidonic acid derivatives by soybean LOX-1 involves an inverse substrate orientation, this conclusion may not be extended to other LOX isoforms. For the positional specificity of the human 5-LOX, which converts arachidonic acid predominantly to 5-HPETE, two alternative hypotheses are currently discussed: (i) the orientation theory suggesting that the substrate may slide into the active site with its carboxylate ahead (*35, 36*) and (ii) the volume theory which postulates a conserved fatty acid orientation for all LOX isoforms. The positional specificity of 5-LOXs may be due to their voluminous substrate binding pocket, and this may allow a substrate alignment that is optimal for 5-lipoxygenation (*32, 33*). For the time being, there is no experimental proof for either of these theories, and their pros and cons have been discussed in detail (*33–39*). The recent finding that the human 5-LOX can be converted to a 15-lipoxygenating enzyme species, when the volume of the substrate binding pocket is reduced (*40*), may add some points to the volume theory. On the other hand, site-directed mutagenesis studies, in which the cucumber lipid body LOX is converted from a linoleate (13*S*)-LOX to a (9*S*)-lipoxygenating enzyme species, suggested an inverse substrate orientation as a structural basis of these alterations (*41*). In this case, mutagenesis may lead to a demasking of a charged amino acid residue at the active site which may strongly reduce the energetic barrier associated with burying the free carboxylate in the substrate binding cleft.

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