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Oxygenation of Trans Polyunsaturated Fatty Acids by Lipoxygenase Reveals Steric Features of the Catalytic Mechanism†

Max O. Funk, Jr.,* Jon C. Andre,‡ and Tetsuo Otsuki§
Department of Chemistry, University of Toledo, Toledo, Ohio 43606
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ABSTRACT: Lipoxygenase, a nonheme iron dioxygenase, catalyzes the oxygenation of 1,4-diene units in polyunsaturated fatty acids, forming conjugated diene hydroperoxides as the primary products. The naturally occurring all-*Z* geometry for the olefins in the polyunsaturated fatty acid has long been thought to be a substrate requirement for the enzyme. A rigorous test of this hypothesis using the two isomeric (9*E*,12*Z*)- and (9*Z*,12*E*)-9,12-octadecadienoic acids was carried out. Both isomeric substrates were found to be catalytically oxygenated by soybean lipoxygenase 1 at a significant fraction of the rate of the reaction of the natural substrate, linoleic acid. Product determinations revealed that a thermodynamically unfavorable *E* to *Z* isomerization at the 9,10-position occurred when (9*E*,12*Z*)-9,12-octadecadienoic acid was converted into the 13-hydroperoxide by lipoxygenase 1. Determination of the stereochemistry at the oxygenated position in the products indicated that a comparable isomerization at the 12,13-position did not occur when the 9*Z*,12*E* isomer was employed. The distribution of products obtained from oxygenation at the 9-position supported the hypothesis that the enzyme catalyzes the reaction in one of two substrate orientations, conventional and head to tail reversed. The observations can be understood on the basis of the steric demands on intermediates in the proposed mechanism of action as well as by catalysis by the active-site iron atom.

The enzyme lipoxygenase is a nonheme iron dioxygenase that plays a major role in polyunsaturated fatty acid metabolism in both plants and animals. In plants, it has recently been demonstrated that lipoxygenase catalysis is part of a biosynthetic sequence leading to growth regulatory substances (Vick & Zimmerman, 1984). In animals, the enzyme catalyzes the inaugural step in the conversion of arachidonic acid into the leukotrienes, a family of compounds with various potent physiological activities (Samuelsson, 1983). The enzyme from soybeans has received the most attention in terms of a detailed physical and chemical characterization because of its early discovery (Theorell et al., 1947), abundance, ease of isolation, and stability (Finnazzi-Agro et al., 1973).

The oxygenation of polyunsaturated fatty acids catalyzed by lipoxygenase 1 from soybeans has long been thought to be specific for substrates containing the (*Z*,*Z*)-1,4-pentadiene structural unit at an appropriate location in the carbon chain (Holman et al., 1968). While the positional specificity of the enzyme for different polyunsaturated fatty acids and the composition of products obtained from these oxygenations have been extensively studied (Hamberg & Samuelsson, 1967), the

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* Address correspondence to this author.
‡ Present address: Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.
§ Present address: Department of Chemistry, Occidental College, Los Angeles, CA 90041.

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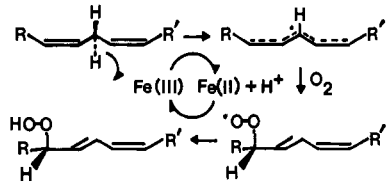
Scheme I

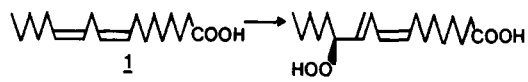


R=$(CH_2)_4CH_3$

R'=$(CH_2)_7COOH$

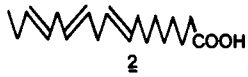
requirement for the all-*Z* geometrical arrangement in the substrate has not been thoroughly documented. In one of the few reports on this subject in the literature, it was found that, in contrast to the natural substrate linoleic acid (**1**), fatty acids

Substrate

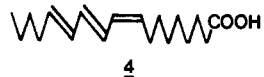


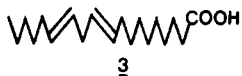
1

Nonsubstrates



2





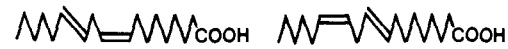
3

4

containing unsaturation in the *E* geometry, linolelaidic, elaidolinolenic, and α-eleostearic acids (**2**–**4**), were not catalytically oxygenated by the enzyme (Holman & Burr, 1945). This experiment does not, however, represent a rigorous test of the hypothesis that the all-*Z* geometry is a substrate requirement of the enzyme. The relevant substrates, **2** and **3**, contained *E* unsaturation at both positions in the 1,4-pentadienyl system. The only compound containing both *E* and *Z* geometry in the same molecule, **4**, was completely conjugated and therefore incapable of being oxygenated by lipoxygenase.

The regiospecificity and stereospecificity of lipoxygenase 1 acting on the natural substrate linoleic acid has been thoroughly characterized. Oxygenations at the pH 9 optimum for the enzyme resulted in the formation of 9- and 13-hydroperoxides in a 3:97 ratio. At pH 6.6, the 9/13 ratio changed to 23:77. The configuration at the oxygenated position was found to be predominantly, although not exclusively, *S* under all conditions (Van Os et al., 1979). The tendency for lipoxygenases to produce hydroperoxides of *S* absolute configuration from natural substrates is apparently quite general (Corey et al., 1980). It has been further demonstrated for lipoxygenase 1 that hydrogen atom abstraction at position 11, the rate-determining step in the catalytic mechanism (Egmond et al., 1973), also occurs stereospecifically. An antarafacial relationship between hydrogen atom abstraction and oxygen addition was found irrespective of the position in the fatty acid oxygenated (Egmond et al., 1972). This was explained in terms of a head to tail reversal of the position of the substrate in the active site of the enzyme. In this way, the enzyme would be expected to produce hydroperoxides of the same configuration at both positions by acting on the substrate in the two alternative orientations in the same way. In Scheme I the current working hypothesis for the mechanism of action of the enzyme is illustrated.

We report here on a rigorous test of the all-*Z* substrate geometry requirement for lipoxygenase 1 using the isomeric (*Z*,*E*)- and (*E*,*Z*)-9,12-octadecadienoic acids (**5** and **6**). Contrary to the long-held view, these compounds were found to be catalytically oxygenated by the enzyme. Additionally,



5 **6**

the regiochemical and stereochemical outcomes of these transformations were highly informative with respect to the steric features of the mechanism of the enzyme-catalyzed reaction.

Experimental Procedures

Materials. Lipoxygenase 1 was obtained from soybeans, cv. Provar, by extraction, differential ammonium sulfate precipitation, dialysis, and chromatofocusing (Funk et al., 1985). (9*Z*,12*E*)-9,12-Octadecadienoic acid (**5**) and (9*E*,12*Z*)-9,12-octadecadienoic acid (**6**) were prepared by total synthesis (Otsuki et al., 1986). The isomeric polyunsaturated fatty acids were judged to be greater than 99% pure by ^{13}C NMR spectroscopy.

Methods. Initial rate studies were carried out in either 0.1 M borate buffer at pH 9.0 (Axelrod et al., 1981) or 0.1 M phosphate at pH 7.0 in the presence of Tween 20 (Grossman & Zakut, 1979). Incubations for the product isolation studies were carried out for 30 min in phosphate buffer (0.1 M, pH 7.0, 0.1% Tween 20) at 25 °C. The substrate concentration in each case was 1 mM. The enzyme concentration was either 25 nM for linoleic acid or 1000 nM for the isomers. The products were isolated by solid-phase extraction (J. T. Baker 7020-1) following acidification to pH 3 using phosphate buffer (0.2 M, pH 2.0). The Tween 20 was removed from the extractor by successive washing with water (2×), while the hydroperoxy polyunsaturated fatty acids were eluted in methanol. The solvent was removed, and the products were reduced, esterified, and purified by chromatography on silica gel as previously described (Porter et al., 1979). The distribution of regioisomers among the products was determined chromatographically (Chan & Levett, 1977). The stereochemistry of products was determined by a recently developed chromatographic technique (Andre & Funk, 1986).

Results

Kinetic Studies. The isomeric octadecadienoic acids were compared with linoleic acid with respect to their ability to act as substrates for lipoxygenase 1 catalysis under standard conditions. When assays were carried out at relatively high enzyme concentration (>10 nM) in borate buffer at pH 9, slight activity was detected for both of the isomers as a slow increase in absorbance at 234 nm due to formation of a conjugated hydroperoxide. This observation eliminated the possibility of significant contamination of the isomers by linoleic acid, since under these conditions the natural substrate would have been completely converted into hydroperoxide during the mixing time of the experiment. The magnitude of the initial increase in absorbance at 234 nm in these experiments provided an upper estimate of the contribution of linoleic acid to the solutions of the isomers at 0.1% of the polyunsaturated fatty acid composition.

Lipoxygenase activity has also been commonly determined in phosphate buffer at pH 7 by using the surfactant Tween 20 as a solubilizing agent for the polyunsaturated fatty acid (Grossman & Zakut, 1979). Under these conditions, the two geometrical isomers were catalytically oxygenated by lipoxygenase 1 at a readily detectable rate. The ratio of the initial rate of oxygenation for each isomer with respect to that for linoleic acid under identical conditions was 0.030.

Product Distribution—Regiochemistry. Incubations of linoleic acid and the two geometrical isomers were carried out under the conditions of the pH 7 assay on a large scale to allow

Table I: Product Distribution for the Oxygenation[a] of Isomeric 9,12-Octadecadienoic Acids (1 mM) by Soybean Lipoxygenase 1 (*Z,Z* 25 nM; *E,Z* and *Z,E* 1000 nM) at pH 7.0 (0.1 M Phosphate, 0.1% Tween 20) at 25 °C

	product			
	13-hydroperoxide		9-hydroperoxide	
substrate	9*Z*,11*E*	9*E*,11*E*	10*E*,12*Z*	10*E*,12*E*
(*Z,Z*)	0.74	0.04	0.18	0.04
(9*E*)	0.55	0.17	0.19	0.09
(12*E*)	0.46	0.18	0.08	0.28

[a] Oxygenations carried out in triplicate with product determinations for each carried out in triplicate; SD <3%.

for the isolation of and the assignment of structure for the products. The hydroperoxides were extracted, reduced with sodium borohydride, and converted into their methyl ester derivatives for high-performance liquid chromatography (HPLC) determination. A comparison of the traces obtained for the product distribution from the three isomeric substrates is presented in Figure 1. It is noteworthy that the same predominant product, (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid, was obtained for each of the isomers. While this was the expected result for the natural *Z,Z* substrate (chromatogram A) and for the 9*Z*,12*E* isomer (chromatogram B), the observation of this product from the 9*E*,12*Z* isomer requires that a thermodynamically unfavorable *E* to *Z* rearrangement at the 9-position accompanies the oxygenation. The relative yields of the isomeric products obtained for the oxygenations of the three polyunsaturated fatty acids are collected in Table I.

With linoleic acid, lipoxygenase 1 catalysis at pH 7 in the presence of Tween 20 gave a product composition very comparable to that previously obtained at pH 6.6 in the absence of the surfactant, 9/13 ratio of 22:78 vs. 23:77, respectively (Van Os et al., 1979). The stereochemical outcome for the natural substrate was for the observation of slightly less specificity in the presence of the surfactant than in its absence, *S*/*R* ratios of 80:20 vs. 93:7, respectively. These observations indicated that the regiochemical and stereochemical outcomes for the enzyme-catalyzed reaction were only slightly affected by the presence of the Tween 20 in the case of linoleic acid as substrate.

Product Distribution—Stereochemistry. The stereochemical outcome of the oxygenations at positions 9 and 13 resulting in products containing *E,Z* geometry was determined chromatographically (Andre & Funk, 1986). The values of the relative yields of *R* and *S* configurations are collected in Table II. It is readily apparent that a distinctive pattern of absolute configuration was obtained for each of the substrates. The interesting feature of these observations was that while the natural substrate was invariably converted into product with mostly *S* configuration, the isomers were converted into certain products with predominantly *R* configuration.

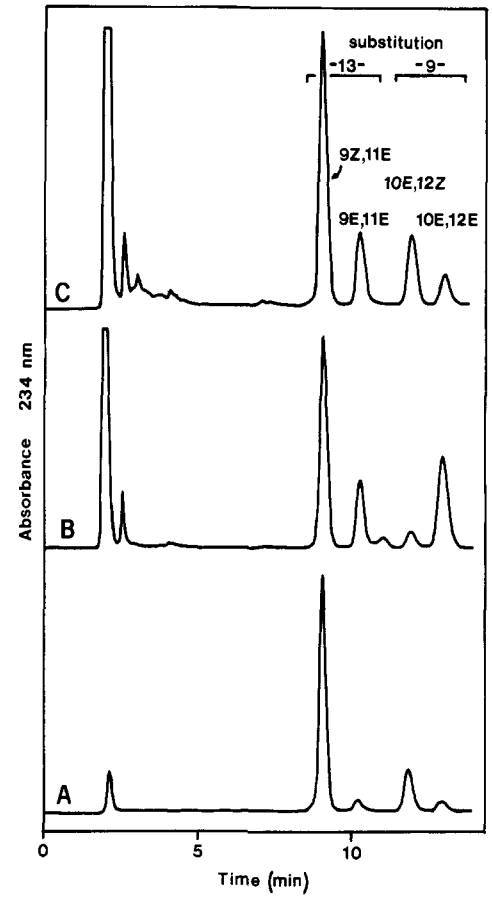
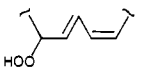
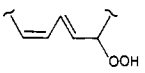
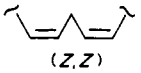
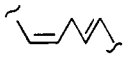


FIGURE 1: Product distributions for the oxygenation of three isomeric 9,12-octadecadienoic acids by soybean lipoxygenase 1, pH 7.0, in 0.1 M phosphate and 0.1% Tween 20 at 25 °C. Determinations were carried out by reversed-phase HPLC of the corresponding alcohols as their methyl ester derivatives. (A) (9*Z*,12*Z*)-9,12-Octadecadienoic acid; (B) (9*Z*,12*E*)-9,12-octadecadienoic acid; (C) (9*E*,12*Z*)-9,12-octadecadienoic acid.

Discussion

The observations reported here represent the discovery of a new element of the substrate specificity of soybean lipoxygenase 1, the oxygenation of polyunsaturated fatty acids containing *E* geometry at one of the unsaturated positions. The rate of the reaction for the two isomers was small compared to that for the natural substrate, 3%, but certainly significant. It is interesting to note that the rates of the oxygenation of the two isomeric substrates were the same. This means that the effect of the *E* geometry on the rate-determining hydrogen atom abstraction did not depend on the position in the chain occupied by the isomeric unsaturation. It was further found that the regiochemical and stereochemical outcomes of the oxygenations of the isomeric substrates provided considerable

Table II: Product Stereochemistry for the Oxygenation[a] of Isomeric 9,12-Octadecadienoic Acids (1 mM) by Soybean Lipoxygenase 1 (*Z,Z* 25 nM; *E,Z* and *Z,E* 1000 nM) at pH 7.0 (0.1 M Phosphate, 0.1% Tween 20) at 25 °C

	product	
	13-hydroperoxide	9-hydroperoxide
		
substrate	9*Z*,11*E*, *R*:*S*	10*E*,12*Z*, *R*:*S*
 (*Z,Z*)	20:80	20:80
 (9*E*)	9:91	63:37
 (12*E*)	67:33	58:42

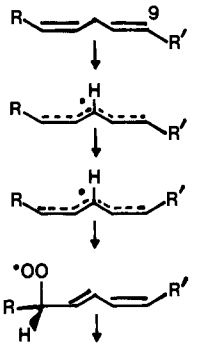
[a] Oxygenations carried out in triplicate with product determinations for each carried out in triplicate; SD <3%.

insight into the steric features of the catalytic mechanism of the enzyme.

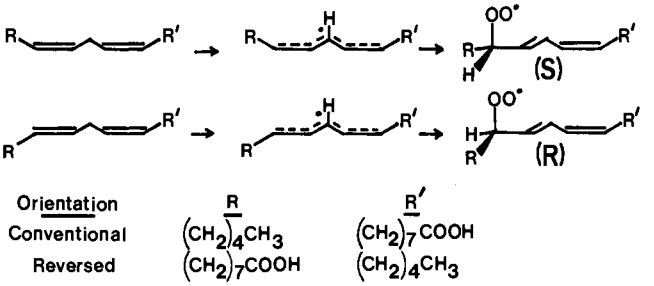
As has been previously demonstrated, it was found in this study that lipoxygenase 1 catalysis of the oxygenation of linoleic acid at pH 7 does not display complete regiospecificity or stereospecificity. This is most probably due to the reversible dissociation of intermediates from the enzyme. Loss of either the pentadienyl radical or the peroxy radical from the enzyme would be expected to lead to the formation of isomeric hydroperoxide products by known nonenzymatic processes (Porter & Wujek, 1984). For example, a pentadienyl radical released from the enzyme would be expected to be oxygenated in a stereorandom fashion at each end of the unsaturated system. The resulting peroxy radicals could undergo bond rotation and loss of oxygen to give isomeric pentadienyl radicals. Direct dissociation of peroxy radicals from the enzyme followed by loss of oxygen would also result in this outcome. Reoxygenation of the isomerized pentadienyl radicals would lead to a mixture of stereoisomeric and regioisomeric peroxy radicals. The peroxy radicals could then be converted into product hydroperoxides by reassociation with and reduction by the enzyme. Clearly, the product composition represents a competition between the rates of a variety of chemical and enzymatic reactions. We interpret the observation that certain transformations are catalyzed by the enzyme in a regiospecific and stereospecific fashion to mean that a portion of the process occurs without dissociation of these intermediates. The resulting trends in the stereochemical and regiochemical outcomes can be used to understand the details of the catalytic mechanism employed by the enzyme.

One striking observation among the product distribution data was the thermodynamically unfavorable *E* to *Z* rearrangement that accompanied the oxygenation of the 9*E*,12*Z* substrate at position 9 upon formation of the 13-hydroperoxide. It is reasonable to assume that this transformation takes place at the stage of the intermediate pentadienyl radical where much of the barrier to rotation of the double bond has been relieved (Scheme II). The energetic parameters for the isomerization of the pentadienyl radical that would account for the observed product have recently been estimated by using electron spin resonance spectroscopy. The barrier to an interconversion like that required by the observed isomerization was found to be approximately 10 kcal mol^{-1} (MacInnes & Walton, 1985). It is generally thought that steric constraints imposed by enzymes can account for at least 5 kcal mol^{-1} in reducing the activation energy of chemical reactions (Fersht, 1985). Lipoxygenase 1, which contains a catalytically competent iron atom, would be particularly well suited to facilitating this reaction. The 1,4 to 1,3 rearrangement of the parent pentadiene system is catalyzed by nonenzymatic iron with a very low activation energy (Mitchener & Wrighton, 1983). Catalysis of the isomerization of olefins by iron carbonyl complexes under mild conditions has also recently been demonstrated (Fleckner et al., 1984). Further, it has been shown that catalysis of this isomerization by iron containing sterically demanding ligands results in the formation of the thermodynamically less favorable *Z* olefin (Graff et al., 1982). A combination of the steric constraints imposed upon the reaction by the enzyme active site and catalysis by iron would appear to be more than adequate to account for the isomerization of the intermediate pentadienyl radical resulting in the formation of the observed product by lipoxygenase 1.

Scheme II



Scheme III



Orientation	R	R′
Conventional	$(CH_2)_4CH_3$	$(CH_2)_7COOH$
Reversed	$(CH_2)_7COOH$	$(CH_2)_4CH_3$

The observed trends in the stereochemical outcomes of the isomeric substrates were also informative. The oxygenations of linoleic acid and the 9*E*,12*Z* isomer at position 13, for example, resulted in the formation of predominantly the *S* stereochemistry in comparison to the 9*Z*,12*E* compound, which gave predominantly the *R* configuration. This outcome would be expected if the 12,13 bond in the intermediate pentadienyl radical is not readily isomerized (as the 9,10 bond apparently is) during the catalytic mechanism. Hydrogen atom abstraction from the substrate with oxygen addition to the resulting planar pentadienyl system from the opposite side would be expected to produce products with *S* configuration from substrates with 12*Z* geometry and *R* configuration from substrate with 12*E* geometry. This is illustrated in Scheme III.

The observation of the stereospecific oxygenation of linoleic acid catalyzed by lipoxygenase 1 at position 9 has been interpreted as arising from the ability of the enzyme to act on the substrate in two orientations (Van Os et al., 1979). If the substrate was accepted to some extent by the active site in a head to tail reversed orientation, catalysis using the proposed

mechanism would be expected to occur with oxygenation at the 9 position and with formation of product in predominantly the *S* configuration. This interpretation presupposes that the spatial relationship of the olefinic system with respect to the active site was the same in the two orientations. Our observation using linoleic acid as the substrate confirms the earlier finding with respect to the stereochemical outcome at position 9. The 9*E*,12*Z* isomer, however, is transformed mainly into 9-hydroperoxide product with *R* configuration. This observation conforms to the view of the control of the stereochemistry during catalysis of the oxygenation of the isomers introduced above. When the orientation of the 9*E*,12*Z* isomer is reversed in head to tail fashion for oxygen addition at position 9, the geometry of the unsaturation at the oxygenated position is *E*. As demonstrated in Scheme III, this would be expected to result in the formation of product with *R* configuration. The control of stereochemistry in these reactions apparently does not extend to the oxygenation of the 9*Z*,12*E* isomeric substrate at the 9-position. In this case dissociation of the intermediates resulting in the formation of isomeric products apparently competes favorably with enzyme catalysis such that mixtures of products reflecting the outcome of nonenzymatic reactions are obtained. The product that would have arisen from the *E* to *Z* rearrangement corresponding to that obtained for oxygenation at the 13-position was obtained as a minor (8%) component. This observation and the reduced stereospecificity found for oxygenation at the 9-position for the isomers generally point to the likelihood of a difference in the dissociation constants for reaction intermediates in the two alternative orientations.

The picture that emerges from these studies on the oxygenations of the isomeric 9,12-octadecadienoates by lipoxygenase 1 is that of an enzyme capable of exerting considerable regiochemical and stereochemical control on the reaction at the level of the pentadienyl radical intermediate. The tendency for lipoxygenase 1 to form 13-hydroperoxides with 9*Z* geometry even when 9*E* geometry is present in the substrate indicates that the enzyme can cause rearrangement to occur at the 9,10-position. It seems quite likely that the steric requirements of the active site as well as catalysis by enzymatic iron are responsible for this transformation. In contrast, stereochemical features of the product distributions of the oxygenations of the isomeric substrates indicate that the similar 12,13 bond of the intermediate pentadienyl radical is not susceptible to isomerization during catalysis.

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