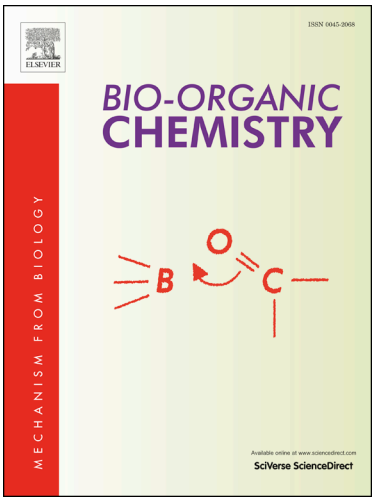


N-Linoleoylamino Acids as Chiral Probes of Substrate Binding by Soybean Lipoxygenase-1

Charles H. Clapp, Justin Pachuski, Natasha F. Bassett, Kathleen A. Bishop, Gillian Carter, Megan Young, Thomas Young, Yuhan Fu

PII: S0045-2068(17)30983-5
DOI: https://doi.org/10.1016/j.bioorg.2018.03.010
Reference: YBIOO 2300

To appear in: *Bioorganic Chemistry*

Received Date: 6 January 2018
Revised Date: 25 February 2018
Accepted Date: 7 March 2018

Please cite this article as: C.H. Clapp, J. Pachuski, N.F. Bassett, K.A. Bishop, G. Carter, M. Young, T. Young, Y. Fu, N-Linoleoylamino Acids as Chiral Probes of Substrate Binding by Soybean Lipoxygenase-1, *Bioorganic Chemistry* (2018), doi: https://doi.org/10.1016/j.bioorg.2018.03.010

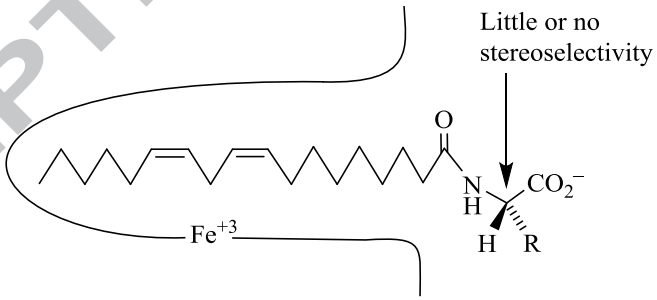
N-Linoleoylamino Acids as Chiral Probes of Substrate Binding by Soybean Lipoxygenase-1

Charles H. Clapp*, Justin Pachuski, Natasha F. Bassett, Kathleen A. Bishop, Gillian Carter, Megan Young, Thomas Young, and Yuhan Fu.

Department of Chemistry, Bucknell University, Lewisburg, PA. 17837

*Corresponding author: cclapp@bucknell.edu

Declarations of interest: none



Graphical Abstract

Highlights

- Soybean lipoxygenase (SBLO-1) oxygenates N-linoleoyl-L-valine (LLV), N-linoleoyl-D-valine (LDV) N-linoleoyl-D-tryptophan (LDT), and N-linoleoyl-L-tryptophan (LLT) primarily at carbon-13 of the linoleoyl moiety.
- Values of k_{cat}/K_m for LLV and LDV are identical within error.
- LDT is a better substrate than LLT by about a factor of 3.
- The absence of stereoselectivity with LLV and LDV and the modest magnitude of the stereoselectivity with LLT and LDT suggest that the amino acid moiety interacts only weakly with the protein.

Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CMC, critical micelle concentration; ENDOR, electron-nuclear double resonance; EPR, electron paramagnetic resonance; ESI, electrospray ionization; GC/MS, gas chromatography/mass spectrometry; HPLC, high pressure liquid chromatography; 9-HODE, 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HODE, 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; HRMS, high resolution mass spectrometry; LDT, N-linoleoyl-D-tryptophan; LLT, N-linoleoyl-L-tryptophan; LDV, N-linoleoyl-D-valine; LLV, N-linoleoyl-L-valine; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; SBLO-1, soybean lipoxygenase-1.

N-Linoleoylamino Acids as Chiral Probes of Substrate Binding by Soybean Lipoxygenase-1

Abstract

Lipoxygenases catalyze the oxygenation of polyunsaturated fatty acids and their derivatives to produce conjugated diene hydroperoxides. Soybean lipoxygenase-1 (SBLO-1) has been the subject of intensive structural and mechanistic study, but the manner in which this enzyme binds substrates is uncertain. Previous studies suggest that the fatty acyl group of the substrate binds in an internal cavity near the catalytic iron with the polar end at the surface of the protein or perhaps external to the protein. To test this model, we have investigated two pairs of enantiomeric N-linoleoylamino acids as substrates for SBLO-1. If the amino acid moiety binds external to the protein, the kinetics and product distribution should show little or no sensitivity to the stereochemical configuration of the amino acid moiety. Consistent with this expectation, N-linoleoyl-L-valine (LLV) and N-linoleoyl-D-valine (LDV) are both good substrates with k_{cat}/K_m values that are equal within error and about 40% higher than k_{cat}/K_m for linoleic acid. Experiments with N-linoleoyl-L-tryptophan (LLT) and N-linoleoyl-D-tryptophan (LDT) were complicated by the low critical micelle concentrations (CMC = 6–8 μM) of these substances. Below the CMC, LDT is a better substrate by a factor of 2.7. The rates of oxygenation of LDT and LLT continue to rise above the CMC, with modest stereoselectivity in favor of the D enantiomer. With all of the substrates tested, the major product is the 13(*S*)-hydroperoxide, and the distribution of minor products is not appreciably affected by the configuration of the amino acid moiety. The absence of stereoselectivity with LLV and LDV, the modest magnitude of the stereoselectivity with LLT and LDT, and the ability micellar forms of LLT and LDT to increase the concentration of available substrate are all consistent with the hypothesis that the amino acid moiety binds largely external to SBLO-1 and interacts with it only weakly.

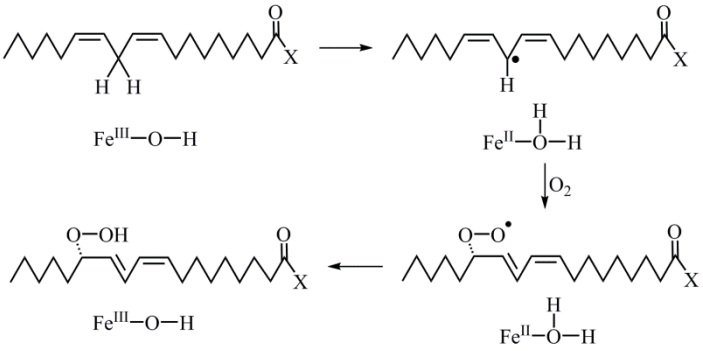
Key Words: Lipoxygenase, specificity, stereoselectivity, substrate binding, N-linoleoylamino acids.

1. Introduction

Lipoxygenases catalyze the oxygenation of *Z*,*Z*-1,4-diene units in polyunsaturated fatty acids and their derivatives to produce conjugated diene hydroperoxides [1,2]. These enzymes are widespread in plants [3] and animals [4], and they also occur in prokaryotes [5]. Lipoxygenases contribute to the synthesis of important signaling molecules, including jasmonic acid [6] in plants and leukotrienes, lipoxins and resolvins in animals [7–9]. Some lipoxygenases act on phospholipids [10–13] and appear to be involved in membrane modification [1,14], storage-lipid mobilization [15], and ferroptosis [16]. Inhibitors of human lipoxygenases are of interest as potential drugs for the treatment of inflammation, asthma, cancer and other disorders [4,7,8,17,18].

Much of our mechanistic insight into lipoxygenases comes from studies on soybean lipoxygenase-1 (SBLO-1). This enzyme catalyzes the oxygenation of linoleate to primarily 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (13-HPOD) [19]. SBLO-1 will also oxygenate other fatty acids, phospholipids and fatty acid derivatives that have a *Z*,*Z* 1,4-diene unit in the ω6 position, and the major product contains oxygen at that position [10–13, 20]. Like most lipoxygenases, SBLO-1 is a nonheme iron protein [21]. The catalytic mechanism (Scheme 1) is thought to involve transfer of a hydrogen atom from the bisallylic carbon of the substrate to a ferric hydroxide species in a process that involves hydrogen tunneling [21–24]. The resulting pentadienyl radical reacts with O_2, with the enzyme likely providing an oxygen channel to control the regio- and stereochemistry [25]. The mechanism postulates that the bisallylic carbon of the substrate binds near the Fe^{+3} ion, and this hypothesis is strongly supported by recent ENDOR studies using samples of linoleate labelled with ^{13}C and SBLO-1 in which the catalytic iron has been replaced by manganese [26].

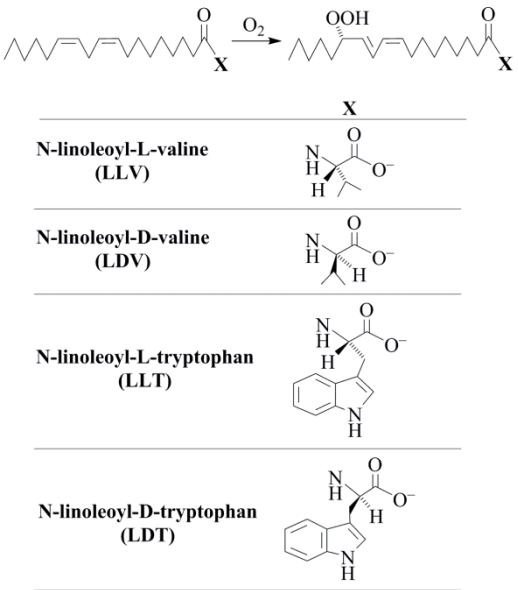
The iron in SBLO-1 is located in the center of a large helical domain [27, 28].



Scheme 1. Reaction mechanism of SBLO-1

The manner in which substrates bind to SBLO-1 and other lipoxygenases in order to position the bisallylic carbon close to the iron has been difficult to establish, and progress towards this goal has been reviewed [29, 30]. The ability of SBLO-1 to oxygenate phospholipids and other substrates with large substituents at the polar terminus is most readily accommodated by the hypothesis that substrates bind with the alkyl tail in an internal cavity close to the iron and the polar terminus near the surface [1, 31]. Binding in this manner would allow large substituents at the polar terminus to remain largely outside the protein. This binding model is supported by recent studies with spin-labelled substrate analogues [29, 32]. The model requires some reorganization of the surface of the protein in order to provide access to the internal cavity, and recent studies suggest that the mobility of helix-2 plays a role in providing this access [33].

Our group has reported that SBLO-1 will oxygenate linoleyltrimethylammonium ion, in which the negatively charged carboxylate group of linoleate is replaced by a positively charged quaternary ammonium group [34]. This finding is consistent with the hypothesis that the polar terminus of substrates has minimal interaction with the protein. As a further test of this model, we have investigated the action of SBLO-1 on the N-linoleoylamino acids shown in Scheme 2. In each case, the major product was determined to be the 13(*S*)-hydroperoxide (Scheme 2); as in the case of linoleate [19], small amounts of other isomeric products were detected. Our goal in these studies was to determine whether the stereochemical configuration of the amino acid moiety affects the kinetics and product distribution. If the amino acid moiety binds external to the protein, the activity of SBLO-1 on these substances is expected to show little or no sensitivity to the configuration of the chiral center. There has been a previous report of activity of SBLO-1 on N-arachidonoylamino acids [35], but the work reported here is the first to focus on the stereoselectivity of the action of SBLO-1 on substrates of this kind.



Scheme 2. Structures of Substrates

2. Materials and Methods

2.1. Materials

SBLO-1 was purified by the method of Axelrod [36], and its concentration was determined using $A^{0.1}_{280nm}$ = 1.6 [37]. Linoleoyl chloride, methyl L-valine hydrochloride, methyl D-valine hydrochloride, methyl L-tryptophan hydrochloride, and methyl D-tryptophan hydrochloride were obtained from Sigma Aldrich. 13(*S*)-HPOD was produced enzymatically from linoleic acid using SBLO-1 and reduced with $NaBH_4$ to give 13(*S*)-hydroxy-9(Z),11(E)-octadecadienoic acid (13(*S*)-HODE). 9(*S*)-HODE, (±)13-HODE, and (±)9-HODE were obtained from Cayman Chemical. Silica gel 60A for flash chromatography was obtained from VWR Scientific.

2.2. Synthesis of N-linoleoyl-L-valine (LLV) and N-linoleoyl-D-valine (LDV)

A solution of 1.00 g (3.35 mmol) of linoleoyl chloride in 5 mL of chloroform was added at room temperature to a stirred mixture of 0.61 g (3.6 mmol) of methyl L-valine hydrochloride and 0.56 g (4.1 mmol) of potassium carbonate in 5.0 mL of chloroform and 5.0 mL of deionized water. The mixture was stirred for 1.25 h. The chloroform layer was separated, and the aqueous layer was extracted with two 10-mL portions of chloroform. The combined chloroform solutions were dried with anhydrous magnesium sulfate and concentrated by rotary evaporation to a colorless oil, which was purified by flash chromatography [38] on a 30 × 3.5 cm column of silica gel using hexanes/ethyl acetate (9:2) to yield an oil with an NMR spectrum consistent with the methyl ester of N-linoleoyl-L-valine. The methyl ester was hydrolyzed by stirring overnight at room temperature with a mixture of 15 mL of 1.0 M aqueous KOH and 15 mL of methanol. The mixture was acidified and extracted with ethyl acetate (3 × 15 mL). The combined ethyl acetate extracts were dried (anhyd. $MgSO_4$) and concentrated to a clear oil. Purification by flash chromatography using hexanes/ethyl acetate/ acetic acid (6:4:0.2) gave a colorless oil with spectroscopic properties consistent with N-linoleoyl-L-valine: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.02 (d, J = 8.4 Hz, 1H), 5.35 (m, 4H), 4.58 (dd, J=8.4 Hz, 4.7 Hz), 2.77 (t, J = 6.0 Hz, 2H), 2.26 (t, J = 7.3 Hz, 2H), 2.23 (m, 1H), 2.04 (m, 4H), 1.64 (t, J = 7 Hz, 2H), 1.3 (m, 14H), 0.99 (d, J = 6.8 Hz, 3H), 0.95 (d, J = 7.0

Hz, 3H), 0.89 (t, J = 6.4 Hz, 3H). ^{13}C NMR (100 Mz, $CDCl_3$) δ: 175.5, 173.9, 130.2, 130.0, 128.1, 127.9, 57.0, 36.7, 31.5, 30.9, 29.6, 29.3, 29.21, 29.18, 29.12, 27.19, 27.18, 25.67, 25.62, 22.6, 19.0, 17.7, 14.0. HRMS (ESI, orbitrap) m/z: $[M-H]^-$: Calcd for $C_{23}H_{40}O_3N$ 378.3014; Found 378.3012.

N-Linoleoyl-D-valine was synthesized by the same method used for LLV and gave identical spectroscopic properties. To assess the stereochemical purity of LLV and LDV, a small sample of each was hydrolyzed, and the resulting valine was derivatized with o-phthaldialdehyde and N-acetyl-L-cysteine, according to the method of Aswad [39]. This derivatization gives different diastereomers (depending on the configuration of the valine), which can be separated by HPLC and detected by fluorescence. Both samples were found to have stereochemical purities of greater than 97% (Figure S1).

2.3. Nonenzymatic Autoxidation of LLV methyl ester

A mixture of the methyl ester of LLV (53 mg, 0.13 mmol) and α-tocopherol (2.6 mg) was incubated at 40°C and periodically flushed with oxygen [40]. After 96 h, the reaction mixture was dissolved in 11.4 mL of ethanol, cooled on ice, and treated with 93 mg of sodium borohydride, which was added in several portions over 5 min with stirring. After 30 min of stirring at 0°C and 30 min at room temperature, the reaction mixture was cooled on ice and treated with acetic acid until gas evolution ceased. Water (13 mL) was added, and the mixture was extracted with diethyl ether (3 × 20 mL). The combined ether extracts were concentrated to a yellow oil that was purified by preparative layer chromatography on a silica gel plate (20 × 20 × 0.1 cm) with fluorescent indicator. The uv-absorbing component at R_f=0.36 was eluted from the plate with ethyl acetate. Evaporation of the ethyl acetate gave an oil, which gave four components on normal-phase HPLC (Figure 4C), two of which have NMR spectra consistent with *E,E* isomers. The appearance of these isomers was unexpected, since the presence of α-tocopherol eliminates the formation of *E,E* isomers with methyl linoleate [40].

2.4. Synthesis of N-linoleoyl-L-tryptophan (LLT) and N-linoleoyl-D-tryptophan (LDT)

These substrates were synthesized from linoleoyl chloride and methyl-L-tryptophan hydrochloride or methyl-D-tryptophan hydrochloride using the procedure described above for the synthesis of LLV.

The methyl esters were purified by flash chromatography using hexanes/ethyl acetate (3:2), and the final products were purified by flash chromatography using hexanes/ethyl acetate/acetic acid (7:11:0.2). ^{1}H NMR ($CDCl_3$, ppm) δ: 8.33 (1 H, s), 7.55 (1 H, d, J = 8.0 Hz), 7.32 (1 H, d, J = 8.4 Hz), 7.17 (1 H, t, J = 7.0 Hz), 7.10 (1 H, t, J = 7.6 Hz), 6.97 (1 H, s), 6.10 (1 H, d, J = 8.0 Hz), 5.35 (4 H, m), 4.92 (1 H, q, J = 6.0 Hz), 3.33 (2 H, m), 2.77 (2 H, t, J = 6.3 Hz), 2.05 (6 H, m), 1.49 (2 H, m), 1.40-1.14 (14 H, m), 0.88 (3 H, t, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$, ppm) δ: 175.3, 174.2, 136.1, 130.3, 130.1, 128.1, 127.9, 127.8, 123.2, 122.2, 119.7, 118.4, 111.4, 109.6, 53.4, 36.4, 31.5, 29.6, 29.3, 29.2, 29.1, 27.2, 27.1, 25.6, 25.4, 22.6, 14.1. HRMS (ESI, orbitrap) m/z: $[M-H]^-$ Calcd for $C_{29}H_{41}O_3,N_2$ 465.3123; Found 465.3128.

2.5. Surface Tension Measurements

Surface tension was determined using an Attension Sigma 703D tensiometer (Bolin Scientific) using a Wilhelmy plate. Solutions were prepared in 50 mM borate, pH 9.0, at least 30 min prior to making measurements. After immersion of the plate in the each test solution, 10 min were allowed to elapse in order for the reading to stabilize.

2.6. Kinetics

Formation of the conjugated diene in the products was monitored spectrophotometrically at 234 nm ($\varepsilon_{234} = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$) at 25°C using either a Beckman DU 640 spectrophotometer (Table 1 and Figure 2) or a Hitachi U2900 spectrophotometer (Table 2). Substrate solutions were prepared in 50 mM sodium borate, pH 9.0, and reactions were initiated by addition of enzyme. The initial rates were determined from the first 10% of the reaction and fit to the Michaelis-Menten equation by nonlinear regression using Sigma Plot.

2.7. Preparative Reaction of LLV with SBLO-1

A 100-mL solution of 132 μM LLV and 3.2 nM SBLO-1 in 50 mM borate, pH 9.0, was stirred vigorously at room temperature until the A_{234} stopped increasing (ca. 15 min). The reaction mixture was acidified to pH 1 with concentrated phosphoric acid and extracted with ether (3 × 20 mL). Concentration of the extracts yielded an oil, which was dissolved in 5.0 mL of ethanol and treated with 11 mg of $NaBH_4$

at 0°C. The mixture was stirred for 30 min at 0°C and 30 min at room temperature, and acetic acid was added cautiously until gas evolution ceased. Water (12 mL) was added, and the solution was extracted with ether (3 × 10 mL). Concentration of the ether extracts yielded an oil, which was dissolved in methanol and treated with ethereal diazomethane (generated from 1-methyl-3-nitro-1-nitrosoguanidine using an Aldrich micro diazomethane generator). Evaporation of the solvents produced an oil. HPLC of this material gave the chromatogram shown in Figure 4 (traces A and B). Approximately 1 mg of this substance was incubated for 1 h at room temperature with 10 μL of pyridine and 10 μL of BSTFA to convert the hydroxyl group to its trimethylsilyl derivative. After evaporation of the pyridine and BSTFA under a stream of nitrogen, the residue was dissolved in 1.0 mL of methanol, and the solution was stirred under H_2 for 1 h with 10 mg of 5% Pd on $CaCO_3$. The catalyst was removed by centrifugation, and the supernatant was analyzed by GC/MS to obtain the data in Figure 3.

2.8. Chromatography

Normal-phase HPLC was carried out using a 250 × 4.6 mm Alltech Adsorbosphere 5μ column eluted isocratically at 1.0 mL/min using the mobile phases indicated in the Results section. UV detection of diene hydroperoxides was carried out at 234 nm. Chiral-phase HPLC was performed on a 250 × 4.6 mm Daicel Chiralpak AD-H column. GC/MS analyses were carried out on a Hewlett/Packard GCD instrument with a 12m × 0.3 mm HPI capillary column. The column temperature was maintained at 50°C for 3 min and then increased from 50–250°C at 20°/min.

2.9. Stereochemical Analysis

The configurations of the carbon-13 oxygenated products from LLV and LDV were determined by the procedure previously described for the products from linoleyldimethylamine [34].

2.10. Hydrolysis of products from LLT and LDT

Oxygenations of LLT and LDT by SBLO-1 and subsequent reduction of the products with $NaBH_4$ were carried out by the same procedure described above for LLV. Approximately 0.5 umole (estimated spectrophotometrically) of unpurified product dissolved in ethanol was added to a reaction vial, and the

ethanol was evaporated under a stream of nitrogen. The residue was dissolved in 1.0 mL of 3.0 M NaOH, and the vial was sealed and heated for 14 h at 120°C. After cooling, the reaction mixture was acidified to pH 3 with phosphoric acid and extracted with two 1-mL portions of dichloromethane. The dichloromethane extracts were concentrated to dryness and dissolved in hexanes for analysis by normal-phase HPLC.

3. Results

3.1. Surface Tension Measurements

Surface-tension measurements were carried out to determine whether the N-linoleoylamino acids in Scheme 2 undergo significant aggregation at the concentrations to be used in the kinetics studies. Figure 1 presents surface tension measurements for solutions of N-linoleoyl-D-valine (0.75–96 μM) and N-linoleoyl-D-trptophan (0.38–48 μM) in 50 mM borate buffer, pH 9.0. Surface tension is plotted against the log of the concentration. The plot for N-linoleoyl-D-valine gives a straight line, which implies that significant aggregation does not occur over this concentration range. Similar results were obtained with N-linoleoyl-L-valine (Figure S2). In contrast, the plot for N-linoleoyl-D-tryptyophan (Figure 1) can be fit to two intersecting lines, and the point of intersection corresponds to a concentration of 6.4 μM, which is taken as the critical micelle concentration (CMC) [41]. Surface tension measurements with N-linoleoyl-L-tryptophan gave a similar plot (Figure S3) with the point of intersection at 7.7 μM. These results indicate that the CMC of the N-linoleoyltryptophan enantiomers is about 6–8 μM.

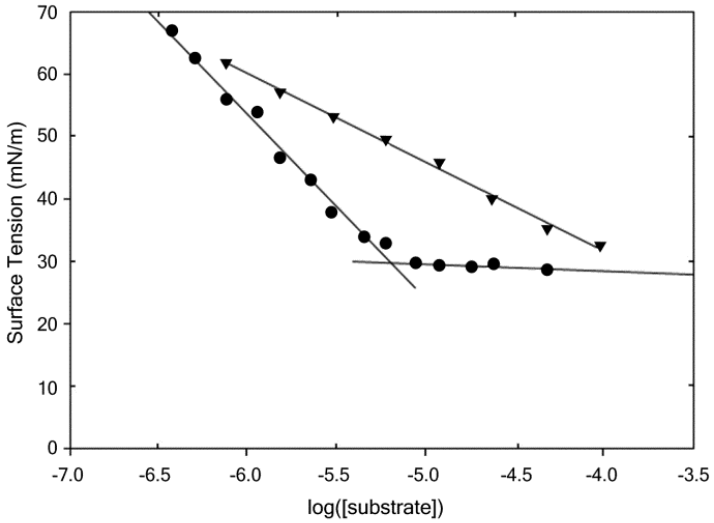


Figure 1. Surface tension of solutions of N-linoleoyl-D-valine (▼) and N-linoleoyl-D-tryptophan (●) in 50 mM sodium borate, pH 9.0.

3.2. Kinetics

Table 1 presents kinetics results for N-linoleoyl-L-valine (LLV), N-linoleoyl-D-valine (LDV) and linoleic acid as substrates for SBLO-1 in 50 mM borate, pH 9.0, at 25°C. Both LLV and LDV are very good substrates, with values of k_{cat}/K_m that are slightly higher than the value for linoleic acid. The kinetic parameters for LLV and LDV are nearly equal, and the values of k_{cat}/K_m are identical within error.

Table 2 presents the initial rates of oxygenation of N-linoleoyl-D-tryptophan (LDT) and N-linoleoyl-L-tryptophan (LLT) at a substrate concentration of 2.0 μM, which is below the critical micelle concentration. Under these conditions, the D enantiomer is a better substrate than the L enantiomer by a factor of 2.7. LDT is also a better substrate at higher concentrations (Figure 2). When the data are fit to the Michaelis-Menten equation, LDT has apparent k_{cat} of 165 ± 8 s^{-1} and an apparent K_m of 5.1 ± 1.1 μM; the corresponding parameters for LLT are 122 ± 7 s^{-1} and 23 ± 3 μM. The data indicate that aggregated forms of LLT and LDT can increase the concentration of available substrate.

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1}, s^{-1})
linoleic acid	220 ± 13	13.3 ± 1.4	16.5 ± 2.1
LLV	205 ± 6	8.9 ± 0.7	23 ± 2
LDV	182 ± 7	7.8 ± 0.7	24 ± 3

Table 1. Kinetic parameters for the oxygenation of substrates by SBLO-1 in 50 mM sodium borate, pH 9.0, 25°C.

	rate $\left(\frac{\mu M}{min}\right)$
LDT	0.82 ± 0.04
LLT	0.31 ± 0.03

Table 2. Initial rates of oxygenation of 2.0 μM LLT and LDT by 2.0 nM SBLO-1 at 25°C in 50 mM sodium borate, pH 9.0

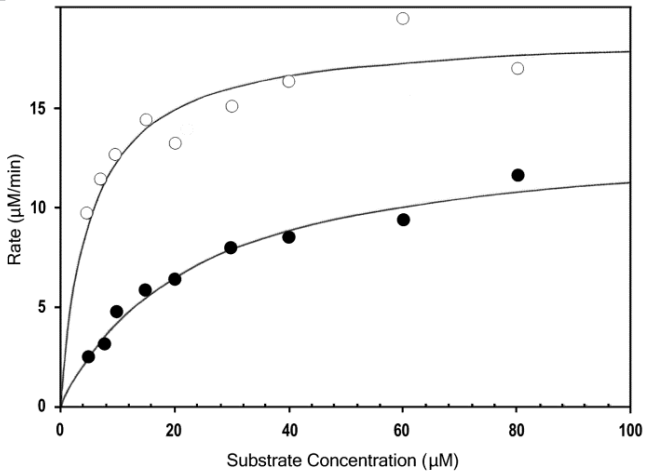


Figure 2. Initial rates of oxygenation of N-linoleoyl-D-tryptophan (○) and N-linoleoyl-L-tryptophan (●) by SBLO-1 (1.9 nM) at 25°C in 50 mm borate, pH 9.0.

3.3. Product Analysis

Incubation of SBLO-1 with LLV gave rise to an absorption at 234 nm characteristic of a conjugated diene, and the intensity of the absorption indicated about 95% conversion to diene product (assuming ε_{234} = 2.5 x 10^4 $M^{-1}cm^{-1}$, the value for 13-HPOD). Following treatment with $NaBH_4$ (to reduce the hydroperoxides to alcohols) and methylation with diazomethane, the NMR spectrum of the product shows the resonances expected for either methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienylvaline or methyl 9-hydroxy-10(*E*),11(*Z*)-octadecadienylvaline. To distinguish between these regioisomers, the crude product was methylated with diazomethane, catalytically hydrogenated, trimethylsilylated, and analyzed by GC/MS. The major product (95%) gave a mass spectrum (Figure 3A) consistent with the presence of a trimethylsiloxy group on carbon-13. A minor product (5%) gave a mass spectrum consistent with the presence of a trimethylsilyloxy group on carbon-9 (Figure 3B). These results indicate that the action of SBLO-1 on LLV produces primarily 13-hydroperoxide.

The methyl esters of the $NaBH_4$-reduced product from LLV were also analyzed by normal-phase HPLC (hexanes/isopropanol, 95:5) with uv detection at 234 nm (Figure 4, traces A and B).

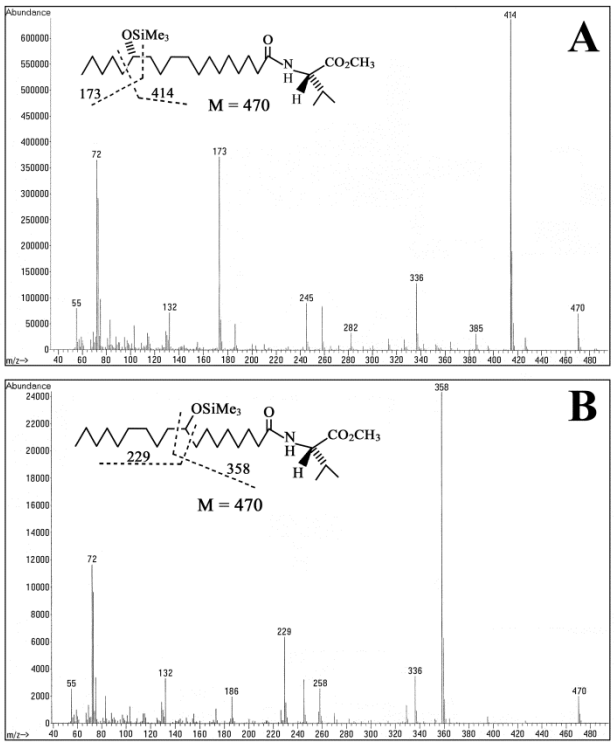


Figure 3. Electron impact mass spectra of derivatized products from N-linoleoyl-L-valine. (A) Major product. (B) Minor product.

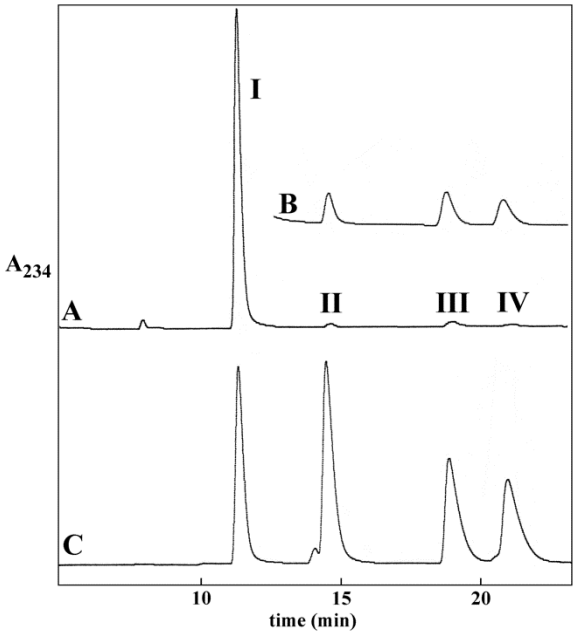


Figure 4. (A) HPLC of the reduced, methylated products the oxygenation of N-linoleoyl-L-valine by SBLO-1. (B) Minor peaks from chromatogram A at higher sensitivity. (C) Reduced products from the nonenzymatic autoxidation of the methyl ester of N-linoleoyl-L-valine.

In addition to the major product (**I**), which eluted at 11 min, minor products (**II–IV**) were detected at 14 min, 19 min and 22 min. The relative intensities of these peaks are shown in Table 3. Four peaks with the same retention times were obtained on a synthetic mixture obtained by nonenzymatic autoxidation of the methyl ester of LLV followed by treatment with $NaBH_4$ (Figure 4, trace C). The four peaks in the synthetic mixture had about equal intensity. NMR spectra of the isolated components of the synthetic mixture indicated that the materials eluting at 11 min and 19 min were *E,Z* isomers and the other two peaks appear to be *E,E*. The presence of small amounts of *E,E* isomers in the product mixture from the oxygenation of linoleic acid by SBLO-1 was previously reported by Gardner [19], and these products have also been detected in our laboratory.

Peak I from the enzymatic reaction was collected and analyzed by chiral-phase HPLC (heptane/ethanol, 80:20, 0.8 mL/min). A major product (98%) was observed at 27 min and a minor product (2%) at 21 min (Figure 5A). A chromatogram of methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienoyl-L-valine from the nonenzymatic autoxidation showed the same two peaks (Figure 5B), but in this case, the intensities were equal, as expected for a racemic mixture. These results indicate that the enzymatic reaction gives primarily one stereoisomer. The configuration at carbon-13 for the

	I	II	III	IV
LLV	92.9 ± 0.3	1.5 ± 0.1	3.1 ± 0.9	2.5 ± 0.6
LDV	95.0 ± 0.9	1.1 ± 0.5	2.7 ± 0.4	1.3 ± 0.1

Table 3. Relative intensities of the HPLC peaks from the analysis of products from oxygenation of linoleoyl-L-valine (LLV) and linoleoyl-D-valine (LDV). **I**–**IV** refer to the labeled peaks in Figure 4. Values are the average of two experiments (± average deviation). The samples of LLV and LDV used in these experiments were freshly purified by flash chromatography and shown by UV spectrometry to contain < 1% diene hydroperoxide. No products were detected in a control experiment from which SBLO-1 was omitted.

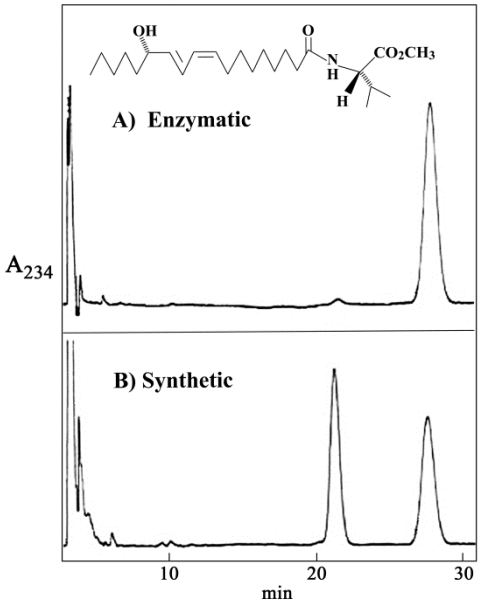


Figure 5. Chiral-phase HPLC of the methyl ester of 13-hydroxy-9Z,11E-octadecadienynoyl-L-valine. (A) Sample produced by oxygenation of N-linoleoyl-L-valine by SBLO-1 followed by reduction with sodium borohydride, methylation with diazomethane and purification by normal phase HPLC. (B) Sample produced by nonenzymatic autoxidation of the methyl ester of N-linoleoyl-L-valine by SBLO-1 followed by reduction, methylation and HPLC purification.

major enantiomer was determined to be *S* using a previously published method [34], as described in the Supplementary Content.

Using the methods described above, the major product obtained from LDV was shown to be 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoyl-D-valine. HPLC analysis of the methylated, $NaBH_4$-treated product mixture gave a product distribution that was similar to that obtained from LLV (Table 3), and chiral-phase HPLC of the major product indicated that the 13(*S*):13(*R*) ratio was 97:3. These results indicate that the stereochemical configuration of the valine moiety does not appreciably affect the products produced by SBLO-1.

Oxygenation of LLT by SBLO-1 followed by reduction with $NaBH_4$ and methylation gave one major product (retention time = 13.9 min) by normal-phase HPLC (hexanes/isopropanol (93:7), 1.0 mL/min). The ^{1}H-NMR spectrum of the HPLC-purified methyl ester was consistent with either the 13-hydroxy or 9-hydroxy products. The trimethylsilyl derivative of the methyl ester was not sufficiently volatile for GC/MS analysis. To establish the regiochemistry of the product, the amide bond was cleaved by alkaline hydrolysis. Analysis of the hydrolysis products by normal-phase HPLC (hexanes/isopropanol/acetic acid, 96:4:0.1) revealed a peak with the same retention time (5.9 min) as 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HODE) and only a very small peak (< 5% of the major peak) at the retention time (7.7 min) of 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HODE). The hydrolysis products were esterified with diazomethane and subjected to chiral-phase HPLC (hexanes/ethanol, 95:5). This gave peaks at the retention times of methyl 13(*S*)-HODE (9.4 min) and methyl 13(*R*)-HODE (7.6 min) in a ratio of 93:7. These results establish that the major product from oxygenation of LLT by SBLO-1 is the C-13 hydroperoxide and has the *S* configuration. Analogous experiments with LDT indicated that the oxygenation occurred primarily (95%) at carbon-13 with a 13(S):13(R) ratio of 94:6.

4. Discussion

SBLO-1 shows little or no stereoselectivity towards the two enantiomers of N-linoleoylvaline. The values of k_{cat}/K_m for LLV and LDV are identical within error, and the distributions of regioisomeric and

stereoisomeric products are not appreciably different. For both enantiomers, the major product is the 13(*S*) hydroperoxide, as in the case with linoleic acid. This result is consistent with the hypothesis that the linoleoyl moieties of LLV and LDV bind in the same manner as linoleic acid.

The k_{cat}/K_m values for the N-linoleoylvaline derivatives are about 40% higher than the value for linoleic acid, owing to a reduction in K_m. These results suggest that the valine moiety makes a small favorable contribution to the affinity for the enzyme. The fact that addition of the valine moiety increases rather than decreases k_{cat}/K_m is most readily accommodated by the hypothesis the valine moiety binds largely external to the protein. The modest size of the increase and the lack of stereoselectively suggest that the interaction of the valine group with the protein is weak.

Reactions catalyzed by SBLO-1 are thought to proceed through a pentadienyl radical bound at the active site (Scheme 1). Differences in the manner in which this intermediate is bound might influence its reactions with O_2 in a manner that affects the distribution of regioisomers or stereoisomers that are formed. The similar product ratios for the LLV and LDV indicate that the stereochemical configuration of the valine moiety has little effect on the binding of the intermediates. With both LLV and LDV, minor products are formed with the same retention times as the *E,E* isomers. The appearance of these isomers suggests that the pentadienyl radical might dissociate from the enzyme on a small fraction of turnovers and react reversibly with O_2 in solution [42]. If dissociation of the intermediate occurs, it does so to about the same extent with both enantiomers, since the percentages of these minor products are not appreciably different.

In order to explore the effects of an amino acid moiety with a larger side chain, we investigated the L and D enantiomers of N-linoleoyltryptophan. These studies were complicated by the finding that these substances have a relatively low critical micelle concentration. Below the CMC, LDT is a better substrate than LLT by a factor of 2.7. For a reaction proceeding through a transition state, a rate difference of this magnitude would indicate that the enzyme binds the transition state for the oxygenation of LDT more favorably than the transition state for oxygenation of LLT by 0.6 kcal/mol. Since the reaction catalyzed

by SBLO-1 involves tunneling, this interpretation is not strictly applicable, but it serves to indicate that the effect of the stereochemical configuration of the tryptophan moiety is small. Interpretation of this difference by modeling is likely to be challenging, since as discussed below, the most likely binding site for the tryptophan moiety is near helix-2, which has been shown to have considerable conformational mobility [33].

Taken together, the results imply that the interaction of SBLO-1 with the amino acid moieties in the substrates we have studied is weak and not highly sensitive to stereochemistry. The results are consistent with the hypothesis that the amino acid moiety binds externally to the enzyme with limited interaction with the surface of the protein. This interpretation also accommodates our finding that the rates of oxygenation of LLT and LDT continue to rise above the CMC. Aggregated forms of these substances could associate with the surface of the enzyme and allow the linoleoyl side chain to enter the active site. Interestingly, the small degree of stereoselectivity in favor of monomeric LDT is still observed in the aggregated forms.

Gaffney and coworkers have studied the interaction of SBLO-1 with substrate analogues that contain a spin label at the polar end [32]. EPR studies of the complexes formed by these spin labels with the enzyme indicate that the polar ends bind at the surface of the protein in a region at the interface of helix-2 and helix-11. The polar groups bearing the spin labels appear to have a high degree of mobility [32,43]. This finding suggests that the polar groups in these substrate analogues interact only weakly with the surface. The results in this study are consistent with the hypothesis that N-linoleoylamino acid substrates bind in a similar manner.

Acknowledgements

We are grateful to Erik Ralph for contributing to the development of some of the methods used in this study. This work was supported by a grant from the National Science Foundation (RUI CHE-1213262) and by the Stephen G. Hobar Memorial Research Fund, the Drs. Anthony and Joyce D. Kales Undergraduate Research Fund, and the Bucknell Program for Undergraduate Research.

References.

[1] A.R. Brash, Lipoxygenases: Occurrence, functions, catalysis, and acquisition of substrate, J. Biol. Chem. 274 (1999) 23679–23682.

[2] I. Ivanov, D. Heydeck, K. Hofheinz, J. Roffeis, V.B. O'Donnell, H. Kühn, M. Walther, Molecular enzymology of lipoxygenases, Arch. Biochem. Biophys. 503 (2010) 161–174.

[3] A. Andreou, I. Feussner, Lipoxygenases – Structure and reaction mechanism, Phytochemistry 70 (2009) 1504–1510.

[4] H. Kuhn, S. Banthiya, K. van Leyen, Mammalian lipoxygenases and their biological relevance, Biochim. Biophys. Acta 1851 (2015) 308–330.

[5] S. Banthiya, J. Kalms, E.G. Yoga, I. Ivanov, X. Carpena, M. Hamberg, H. Kuhn, P. Scheerer, Structural and functional basis of phospholipid oxygenase activity of bacterial lipoxygenase from *Pseudomonas aeruginosa*, Biochim. Biophys. Acta 1861 (2016) 1681–1692.

[6] C. Wasternack, E. Kombrink, Jasmonates: Structural requirements for lipid-derived signals active in plant stress responses and development, ACS Chemical Biology 5 (2010) 63–77.

[7] J.Z. Haeggström, C.D. Funk, Lipoxygenase and leukotriene pathways: Biochemistry, biology, and roles in disease, Chem. Rev. 111 (2011) 5866–5898.

[8] O. Rådmark, O. Werz, D. Steinhilber, F. Samuelsson, 5-Lipoxygenase, a key enzyme for leukotriene biosynthesis in health and disease, Biochim. Biophys. Acta 1851 (2015) 331–339.

[9] C.N. Serhan, N.A. Petasis, Resolvins and protectins in inflammation resolution, Chem. Rev. 111 (2011) 5922–5943.

[10] A.R. Brash, C.D. Ingram, T.M. Harris, Analysis of a specific oxygenation reaction of soybean lipoxygenase-1 with fatty acids esterified in phospholipids, Biochemistry 26 (1987) 5465–5471.

[11] J.J. Murray, A.R. Brash, Rabbit reticulocyte lipoxygenase catalyzes specific 12(*S*) and 15(*S*) oxygenation of arachidonoyl-phosphatidylcholine, Arch. Biochem. Biophys. 265 (1988) 514–523.

[12] M. Pérez-Gilabert, G.A. Veldink, J.F.G. Vliegenthart, Oxidation of dilinoleoyl phosphatidylcholine by lipoxygenase 1 from soybeans, Arch. Biochem. Biophys. 354 (1998) 18–23.

[13] L.S. Huang, M.R. Kim, D-E. Sok, Linoleoyl lysophosphatidylcholine is an efficient substrate for soybean lipoxygenase-1, Arch. Biochem. Biophys. 455 (2006) 119–126.

[14] H. Kühn, J. Belkner, R. Wiesner, A.R. Brash, Oxygenation of biological membranes by the pure reticulocyte lipoxygenase, J. Biol. Chem. 265 (1990) 18351–18361.

[15] I. Feussner, C. Wasternack, H. Kindl, H. Kühn, Lipoxygenase-catalyzed oxygenation of storage lipids is implicated in lipid mobilization during germination, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 11849–11853.

[16] S.E. Wenzel, Y.Y. Tyurina, J. Zhao, C.M. St. Croix, H.H. Dar, G. Mao, V.A. Tyurin, T.S. Anthonymuthu, A.A. Kapralov, A.A. Amoscato, K. Mikulska-Ruminska, I.H. Shrivastava, E.M. Kenny, Q. Yang, J.C. Rosenbaum, L.J. Sparvero, D.R. Emlet, X. Wen, Y. Minami, F. Qu, S.C. Watkins, T.R. Holman, A.P. VanDemark, J.A. Kellum, I. Bahar, J. Bayir, V.E. Kagan, PEBP1 wardens ferroptosis by enabling lipoxygenase generation of lipid death signals, Cell 171 (2017) 628–641.

[17] D. Steinhilber, B. Hoffman, Recent advances in the search for novel 5-lipoxygenase inhibitors, Basic Clin. Pharmacol. Toxicol. 114 (2014) 70–77.

[18] D.K. Luci, J.B. Jameson II, A. Yasgar, G. Diaz, N. Joshi, A. Kantz, K. Markham, S. Perry, N. Kuhn, J. Yeung, E.H. Kerns, L. Schultz, M. Holinstat, J.L. Nadler, D.A. Taylor-Fishwick, A. Jadhav, A. Simeonov, T.R. Holman, D.J. Maloney, Synthesis and structure–activity relationship studies of 4-((2-hydroxy-3-methoxybenzyl)amino)benzenesulfonamide derivatives a s potent and selective inhibitors of 12-lipoxygenase, J. Med. Chem. 57 (2014) 495–506.

[19] H.W. Gardner, Soybean lipoxygenase-1 enzymically forms both (9*S*)- and (13*S*)-hydroperoxides from linoleic acid by a pH-dependent mechanism, Biochim. Biophys. Acta 1001 (1989) 274–281.

[20] M. Hamberg, B. Samuelsson, On the Specificity of the Oxygenation of Unsaturated Fatty Acids Catalyzed by Soybean Lipoxidase, J. Biol. Chem. 242 (1967) 5329–5335.

[21] M.O. Funk Jr., R.T. Carroll, J.F. Thompson, R.H. Sands, W.R. Dunham, Role of iron in lipoxygenase catalysis, J. Am. Chem. Soc. 112 (1990) 5375–5376.

[22] M.H. Glickman, J.P. Klinman, Lipoxygenase reaction mechanism: Demonstration that hydrogen abstraction from substrate precedes dioxygen binding during catalytic turnover, Biochemistry 35 (1996) 12882–12892.

[23] T. Jonsson,, M.H. Glickman, S .Sun, J.P. Klinman, Experimental evidence for extensive tunneling of hydrogen in the lipoxygenase reaction: Implications for enzyme catalysis, J. Am. Chem. Soc. 118 (1996) 10319–10320.

[24] J.P. Klinman, A.R. Offenbacher, S. Hu, Origins of enzyme catalysis: Experimental findings for C–H activation, new models, and their relevance to prevailing theoretical constructs, J. Am. Chem. Soc. 139 (2017) 18409–18427.

[25] L. Collazo, J.P. Klinman, Control of the position of oxygen delivery in soybean lipoxygense-1 by amino acid side chains within a gas migration channel, J. Biol. Chem. 291 (2016) 9052–9059.

[26] M. Horitani, A.R. Offenbacher, C.A. Marcus Carr, T. Yu, V. Hoeke, G.E. Cutsail III, S. Hammes-Schiffer, J.P. Klinman, B.M. Hoffman, ^{13}C ENDOR spectroscopy of lipoxygenase-substrate complexes reveals the structural basis for C–H activation by tunneling, J. Am. Chem. Soc.139 (2017) 1984–1997.

[27] J.C. Boyington, B.J. Gaffney, L.M. Amzel, The three-dimensional structure of an arachidonic acid 15-lipoxygenase, Science 260 (1993) 1482–1486.

[28] W. Minor, J. Steczko, B. Stec, Z. Otwinowski, J.T. Bolin, R. Walter, B. Axelrod, Crystal structure of soybean lipoxygenase L-1 at 1.4 A resolution, Biochemistry 35 (1996) 10687–10701.

[29] B.J. Gaffney, Connecting lipoxygenase function to structure by electron paramagnetic resonance, Acc. Chem. Res. 47 (2014) 3588–3595.

[30] M.E. Newcomer, A.R. Brash, The structural basis for specificity in lipoxygenase catalysis, Protein Sci. 24 (2015) 298–309.

[31] G. Coffa, A.N. Imber, B.C. Maguire, G. Laxmikanthan, C. Schneider, B.J. Gaffney, A.R. Brash, On the relationships of substrate orientation, hydrogen abstraction, and product stereochemistry in single and double dioxygenations by soybean lipoxygenase-1 and its Ala542Gly mutant, J. Biol. Chem. 280 (2005) 38756–38766.

[32] B.J. Gaffney, M.D. Bradshaw, S.D. Frausto, F. Wu, J.H. Freed, P. Borbat, Locating a lipid at the portal to the lipoxygenase active site, Biophysical Journal 103 (2012) 2134–2144.

[33] M.D. Bradshaw, B.J. Gaffney, Fluctuations of an exposed π-helix involved in lipoxygenase substrate recognition, Biochemistry 53 (2014) 5102–5110.

[34] L.E. Chohany, K.A. Bishop, H. Camic, S.J. Sup, P.M. Findeis, C.H. Clapp, Cationic substrates of soybean lipoxygenase-1, Bioorg. Chem. 39 (2011) 94–100.

[35] J.J. Prusakiewicz, M.V. Turman, A. Vila, H.L. Ball, A.H. Al-Messtarihi, V. Di Marzo, L.J. Marnett, Oxidative metabolism of lipoamino acids and vanilloids by lipoxygenases and cyclooxygenases, Arch. Biochem. Biophys. 464 (2007) 260–268.

[36] B. Axelrod, T.M. Cheesbrough, S. Laakso, Lipoxygenase from soybeans, Methods Enzymol. 71 (1981) 441–451.

[37] L. Petersson, S. Slappendel, M.C. Feiters, J.F.G. Vliegenthart, Magnetic susceptibility studies on yellow and anaerobically substrate-treated yellow soybean lipoxygenase-1, Biochim. Biophys. Acta 913 (1987) 228–237.

[38] W.C. Still, M. Kahn, A. Mitra, Rapid chromatographic technique for preparative separations with moderate resolution, J. Org. Chem. 43 (1978) 2923–2925.

[39] D.W. Aswad, Determination of D- and L-aspartate in amino acid mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of o-phthaldialdehyde, Anal. Biochem. 137 (1984) 405–409.

[40] K.E. Peers, D.T. Coxon, H.W-S. Chan, Autoxidation of methyl linolenate and methyl linoleate: The effect of α-tocopherol, J. Sci. Food Agric. 32 (1981) 898–904.

[41] J. Verhagen, J.F.G. Vliegenthart, J. Boldingh, Micelle and acid-soap formation of linoleic acid and 13-hydroperoxylinoleic acid being substrates of lipoxygenase-1, Chem. Phys. Lipids 22 (1978) 255–259.

[42] N.A. Porter, B.A. Weber, H. Weenen, J.A. Khan, Autoxidation of polyunsaturated lipids. Factors controlling the stereochemistry of product hydroperoxides, J. Am. Chem. Soc. 102 (1980) 5597–5601.

[43] F. Wu, B.J. Gaffney, Dynamic behavior of fatty acid spin labels within a binding site of soybean lipoxygenase-1, Biochemistry 45 (2006) 12510–12518.