On the Relationships of Substrate Orientation, Hydrogen Abstraction, and Product Stereochemistry in Single and Double Dioxygenations by Soybean Lipoxygenase-1 and Its Ala542Gly Mutant*

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Recent findings associate the control of stereochemistry in lipoxygenase (LOX) catalysis with a conserved active site alanine for S configuration hydroperoxide products, or a corresponding glycine for R stereoconfiguration. To further elucidate the mechanistic basis for this stereocontrol we compared the stereoselectivity of the initiating hydrogen abstraction in soybean LOX-1 and an Ala542Gly mutant that converts linoleic acid to both 13S and 9R configuration hydroperoxide products. Using 11R-3H- and 11S-3H-labeled linoleic acid substrates to examine the initial hydrogen abstraction, we found that all the primary hydroperoxide products were formed with an identical and highly stereoselective pro-S hydrogen abstraction from C-11 of the substrate (97–99% pro-S-selective). This strongly suggests that 9R and 13S oxygénations occur in the active site, and as the equivalent 9R and 13S products were formed from a bulky ester derivative (1-palmitoyl-2-linoleoylphosphatidylcholine), one can infer that the orientation is tail-first. Both the EPR spectrum and the reaction kinetics were altered by the R product-inducing Ala-Gly mutation, indicating a substantial influence of this Ala-Gly substitution extending to the environment of the active site iron. To examine also the reversed orientation of substrate binding, we studied oxygenation of the 15S-hydroperoxide of arachidonic acid by the Ala542Gly mutant soybean LOX-1. In addition to the usual 5S,15S- and 8S,15S-dihydroperoxides, a new product was formed and identified by high-performance liquid chromatography, UV, gas chromatography-mass spectrometry, and NMR as 9R,15S-dihydroperoxyeicosa-5Z,7E,11Z,13E-tetraenoic acid, the R configuration “partner” of the normal 5S,15S product. This provides evidence that both tail-first and carboxylate end-first binding of substrate can be associated with S or R partnerships in product formation in the same active site.

Polysaturated fatty acids are oxygenated to signaling molecules of very specific structure by the dioxygenases lipoxygenase (LOX)4 and cyclooxygenase, and by the cytochrome P450 class of monooxygenase (1–3). One of the fundamental differences lies in activation of the fatty acid by the dioxygenases compared with oxygen activation by the P450s. In the case of P450s, the oxygen is bound to the heme iron, and the control of reaction specificity largely depends on the specific binding of substrate and its presentation to the activated ferryl oxygen (4–6). By contrast, in both classes of dioxygenase the fatty acid substrate is activated to a radical species capable of instantaneous reaction with molecular oxygen (1, 2); all elements of reaction specificity thus depend on arranging the meeting of molecular oxygen with the correct site on the substrate radical. All the evidence suggests that the reacting oxygen molecule is untethered and that other factors in the enzyme active site must determine the reaction outcome. In particular, cyclooxygenase enzymes have no residues capable of direct oxygen binding within the oxygenase active site (7, 8), whereas for lipoxygenases there is both spectroscopic and kinetic isotope data to refute the involvement of the active site nonheme iron in oxygen binding (9–11). The possibility of oxygen channeling through the protein is one potential means of achieving the targeting of O2 onto a particular carbon of the reacting fatty acid radical (12–14). The influence of protein residues in shielding some of the reactive carbons must also figure into the control of product regiochemistry and stereochemistry. These two options relate directly to the studies we report here on the specificity of reaction by soybean lipoxygenase.

Soybean LOX-1 is the prototypical enzyme of its class and is the best-studied model of lipoxygenase catalysis and structure. Mechanistic studies are supported by the availability of two highly resolved x-ray crystal structures (12, 15). Soybean LOX-1 has been used over the years to establish many fundamental concepts of lipoxygenase catalysis. Among these are the high specificity of the reaction and formation of a chiral product (16); the sole product is 13S-hydroperoxyoctadecadienoic acid (13S-HPODE) from linoleic acid, and the corresponding 15S-hydroperoxide from arachidonic acid. Early on it was shown that the specific oxygenation is associated with a highly stereoselective initial hydrogen abstraction from the fatty acid and that the two events occur...
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Experimental Procedures—Mutagenesis primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Mutagenesis was performed according to the manufacturer’s protocol (Stratagene QuickChange Mutagenesis Kit, La Jolla, CA). Plasmids were purified using QIAprep Spin Miniprep kits (Qiagen, Valencia, CA). Stratagene XL1-Blue super competent cells and BL21-Gold(DE3) cells were transformed with the expression vector following the manufacturer’s instructions. Mutant plasmids were identified by sequencing. DNA sequencing was performed at the FSU Biology DNA Sequencing Facility using an Applied Biosystems 3100 Genetic Analyzer.

Cloning and Expression—The plasmid originally constructed from soybean mRNA transcript had three single base mutations that were corrected by mutagenesis to the J02795 coding sequence, and one three-base mutation (AGT to GAG at positions 478–480 encoding a Ser160Glu change) that was retained in the construct for most experiments. Ser160 was introduced for some comparisons, as indicated in the text. The yield of expressed protein was improved by lowering the temperature of incubation after isopropyl 1-thio-β-D-galactopyranoside induction and by lengthening the incubation period, until the final condition of 14 °C for 30 h was determined to be optimal. The isolated protein yield was halved if the cells were harvested after 24 h. The mutant proteins examined in this study were Ala542 → Gly, Ala542 → Ser, Ala542 → Thr, and Ala542 → Val. Yields of the expressed mutant proteins were similar to yields of native lipoygenase: typically 10 mg of lipoygenase/liter of original culture were obtained after purification for wild-type and somewhat less for mutants.

Wild-type and mutant soybean LOX-1 were expressed in E. coli BL21(DE3) Gold (Stratagene). Overnight cultures in LB (37 °C) were diluted 200-fold into multiple 1-liter flasks containing 375 ml of TB. Cultures were grown to A600 > 1.2 (4–5 h, 37 °C), cooled to 14 °C, induced with isopropyl 1-thio-β-D-galactopyranoside (0.1 mM), and grown for 30 h at 8 °C. Cells were harvested and incubated for 30 min in TSE (0.1 M Tris acetate, 0.5 M sucrose, 1 mg/ml lysozyme, and 0.5 mM EDTA, pH 7.6). Pelleted spheroplasts were frozen at −70 °C. Thawed pellets were suspended in BugBuster (Novagen, 60 ml/liter of original culture). After 20 min at room temperature, the suspension was clarified by centrifugation (18,600 × g in this and subsequent steps), and solid ammonium sulfate was added to the supernatant to 30% of saturation. After 20 min, a pellet was removed by centrifugation, the supernatant was brought to 40% ammonium sulfate saturation, and then clarified again by centrifugation, and finally the supernatant was brought to 65% saturated ammonium sulfate and the suspension was centrifuged immediately. The 65% ammonium sulfate pellet from 3 liters of culture was suspended in wash buffer and immediately cycled several times through a prepared column of Ni-NTA (Novagen, 6 ml from 50% saturation). The Ni-NTA was washed with 100 ml of wash buffer, and then His-tagged lipoygenase was eluted at once with wash buffer containing 250 mM imidazole, and dialyzed overnight (4 °C) in Tris-HCl (10 mM, pH 8). The dialyzed eluate from Ni-NTA was separated by anion exchange chromatography (1 × 15 cm DE-52 Whatman column). Fractions containing soybean LOX-1 activity were brought to 65% saturation with ammonium sulfate and centrifuged, and the pellet was suspended in 1–2 ml of HPLC buffer and dialyzed in HPLC buffer. Lipoygenase elutes at ~30 min from the Superdex 200 HR 10/30 HPLC column. Purity was assessed by SDS-PAGE and was >95%.

The amino-terminal His tag in expressed lipoygenases was removed by incubating 10 units of Factor Xa/mg LOX protein for 4 h at room temperature, and the reaction was stopped using iXrest-agarose, according to the manufacturer’s protocol (Novagen, Factor Xa Cleavage Capture Kit). Typically, 5–10 mg of protein was digested in one batch and the yield of cleaved LOX protein, after workup, was >50% of the starting uncleaved protein. Completion of cleavage was verified by SDS-PAGE (7.5% gel). Samples were AMS precipitated, brought up to 5–10 mg/ml, and dialyzed to remove remaining cleaved peptide.

Samples were prepared for metal analysis by centrifugation and dialysis into 0.01 M Tris, 5 μM sodium azide, pH 7.6. The Tris buffer was

on opposite faces of the reacting pentadiene (an antarafacial relationship) (16, 17). Related observations in comparing the 9S-specific corn lipoxygenase with the 13S-specific soybean enzyme led to the important concept of substrate being capable of adopting a reversed orientation of binding in the lipoxygenase active site (18). This reversed orientation hypothesis has remained open to debate in that there is no LOX structure available with bound substrate to directly support or refute the various lines of argument. Indeed, a recent study suggested a head-first orientation of the residue is conserved as an Ala in active site residue in controlling the regio- and stereospecificity of arachidonic acid oxygenation (21). The residue is conserved as an Ala in lipoxygenases that form S-specific hydroperoxides and as Gly in the R-hydroperoxide-forming enzymes. The Ala → Gly mutation in the enzymes studied at least partially switched the position and R or S chirality of oxygenation on the substrate such that an enzyme with original 8R-specificity could be mutated to make 12S product, 1S to 1R, and so on. These changes correspond to a switch in the position of oxygenation from one end of the fatty acid pentadiene intermediate to the other. The aim of the present study was to investigate some novel aspects of the reaction of wild-type and Ala542 → Gly mutant soybean LOX-1, including the specificity of the associated hydrogen abstraction and the characteristics of double dioxygenation as they pertain to our understanding of substrate binding and the stereoccontrol of oxygenation.

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passed through a Chelex column before use. Glassware for analytical samples was washed sequentially with nitric acid and water. Metal analyses by Inductively Coupled Plasma Mass Spectrometry were performed at the Chemical Analysis Laboratory, University of Georgia (Athens, GA). Percent iron values are based on absorbances at 280 nm for a 1 mg/ml protein solution of 1.28 (with His tag) and 1.35 (no tag) (23). The active samples analyzed included one of Ser160/Gly342 without His tag, three of Gly342 with His tag, one of Ser342 with tag, and two of wild type (Glu160) with tag. The samples for metal determination ranged in concentration from 1 to 15 μM LOX protein and iron, nickel, manganese, copper, and zinc were analyzed. To determine which metals were correlated with protein concentration, metal analysis and protein concentration data of all samples were graphed using KaleidaGraph (micromoles of protein). The metal concentrations that correlated with protein concentration (mole ratio metal/protein) were iron (0.62), manganese (0.02), and, weakly correlated, nickel (0.04) (Pearson's R-values of 0.99, 0.96, and 0.69 respectively). Average amounts of zinc (2 μM) and copper (0.2 μM) in the samples were not correlated with protein concentration. Single analyses of iron in threonine and valine mutants with His tags indicated iron incorporation similar to that of the active samples. The dialysis buffer at the final sample preparation step for metal analysis had negligible iron, manganese, nickel, copper, and zinc content.

Assay System for Wild-type and Mutant Soybean LOX-1—Recombinant wild-type soybean LOX-1 or mutants (1 μg) were incubated with either [1-14C]LA or [1-14C]AA (100 μM final concentration) in 100 μl of 50 mM borate buffer, pH 9, 150 mM NaCl for 2 min at room temperature. Incubations were stopped by addition of methanol, and the pH was reduced to ~6 by addition of 1 N HCl prior to product extraction using the Bligh and Dyer method (24). For experiments studying the second oxygenation reactions, soybean LOX-1 and the Ala542 → Gly mutant (50 μg) were incubated with 15S-HETE (10 μg) in 0.5 ml of 50 mM, pH 9 borate buffer. Reactions were monitored in the UV (200–350 nm), and upon completion products were extracted with dichloromethane (0.5 ml) after acidification to ~pH 6 with 1 N HCl. Hydroperoxy derivatives were resuspended in methanol and reduced to hydroxy fatty acids by addition of 3 μl of triphenylphosphine (TPP, 5 mg/ml) prior to HPLC analysis. Wild-type soybean LOX-1 and the Ala542 → Gly mutant (10 μg) were incubated with either C15/LA-PC or C15/AA-PC (100 μM final concentration) in 0.5 ml of 50 mM borate, pH 9, 150 mM NaCl and 10 mM deoxycholate. Reactions were monitored by UV spectrophotometry at 235 nm and stopped by addition of methanol. Products were extracted using the Bligh and Dyer procedure (24), reduced with TPP, and transesterified with sodium methoxide prior to HPLC analysis.

Incubation with Stereospecifically Labeled Linoleic Acids—The stereospecifically labeled 11R-3H and 11S-3H linoleic acids were synthesized previously as described (25). The tritium-labeled linoleates were mixed with [14C]LA, which served as an internal standard for measurement of tritium retention. Incubations were carried out in 1 ml of 50 mM borate, pH 9, 150 mM NaCl using 10,000 cpm 3H for incubations with the wild-type enzyme and 20,000 cpm 3H for experiments with the Ala542 → Gly mutant. The appearance of the conjugated diene chromophore was monitored by UV spectrometry (200–300 nm), and reaction was stopped by addition of methanol at ~80% substrate conversion. The products were acidified and extracted by the Bligh and Dyer procedure (24), reduced with TPP, and methylated with diazomethane prior to HPLC analysis. Fractions were collected and counted for 3H and 14C, and the percentage of 3H retained was estimated by comparison of the 3H/14C ratios of product and substrate.

HPLC Analysis—Products of the reaction of LOX enzymes and mutants with [1-14C]LA, [1-14C]AA, and 15S-HETE were analyzed on an Agilent 1100 HPLC equipped with a diode array detector connected online to a Radiomatic FLO-ONE A-100 radioactive detector. Metabolites from [1-14C]LA were analyzed using a Whatman® Partisil 5 silica column (0.46 × 25 cm) eluted at a flow rate of 1 ml/min with hexane/isopropanol/acetic acid (100/20/0.1, by volume) with UV detection at 235 nm. Metabolites from incubation with [1-14C]AA were analyzed on a Waters Symmetry C18 5-μm column (25 × 0.46 cm) using a solvent of methanol/water/acetic acid (80/20/0.01, by volume) eluted at 1 ml/min with UV detection at 235 nm; the unreacted substrate was eluted with methanol. The oxygenated products of 15S-HETE were eluted with methanol/water/acetic acid (75/25/0.01, by volume) at a flow rate of 1 ml/min. Methyl esters of products of incubations with either C15/LA-PC or stereospecifically labeled linoleic acids were analyzed on a Whatman® Partisil 5 silica column (0.46 × 25 cm) eluted at a flow rate of 1 ml/min with hexane/isopropanol/acetic acid (100/1/0.1 by volume).

HTE and HODE metabolites collected from the HPLC systems above were converted to methyl esters by treatment with ethereal diazomethane and further purified by SP-HPLC prior to chiral phase HPLC analysis. Chiral analysis of the HTE methyl esters was performed using a Daicel Chiralpak AD (0.46 × 25 cm) eluted at a flow rate of 1 ml/min with hexane/methanol (100/2, by volume), HODE methyl esters were eluted with hexane/methanol (100/5, by volume) with UV detection at 235 nm (26).

Determination of 3H Retention in Products Formed from Stereospecifically Labeled Linoleic Acids—HPODE products formed by the wild-type soybean LOX-1 and the Ala542 → Gly mutant were reduced with TPP, converted to the methyl ester derivative with diazomethane and purified by SP-HPLC and chiral phase HPLC as described under “HPLC Analysis.” Fractions of 0.5 ml were collected from the chiral HPLC column directly into scintillation counting vials. The solvent was evaporated under nitrogen, scintillation fluid was added, and the samples each counted for 10 min in a Packard 1900 TR liquid scintillation analyzer using the dual channel mode for 3H and 14C. Typically there were 1000–2000 cpm of 14C in the product peaks, and the samples were counted for 30 min to improve precision. The counts in the baseline fractions adjacent to the chromatographic peaks were used as background. The 3H retention was calculated by comparison of the 3H/14C ratio of substrate and product.

Preparation of 9R,15S-diHETE and 9S,15S-diHETE—Wild-type soybean LOX-1 (50 μg in 1 ml) was incubated with 95- or 9R-HETE (50 μM) prepared by autoxidation of methyl arachidonate, purification by SP-HPLC, resolution as 9R and 9S by chiral chromatography using a Chiralpak AD column (9R elutes before 9S (27)) with a solvent of hexane/methanol 100/2, by volume, and alkaline hydrolysis to give the corresponding free acids (26). To prepare milligram amounts of 9R,15S-diHETE and 9S,15S-diHETE soybean LOX-1 (2 mg, Sigma type V) was incubated with 9S-S-HETE (20 mg) in 100 ml of 50 mM borate buffer, pH 9, 150 mM NaCl, products of reaction were extracted in dichloromethane at pH 6, re-dissolved in methanol, and reduced with NaBH4 prior to methylation with diazomethane. The analysis of the methyl ester trimethylsilyl ether-hydrogenated derivatives of the products was conducted in the positive-ion electron impact mode (70 eV) by using a Hewlett-Packard 5989A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph equipped with an RTX-1701 fused silica capillary column (5 m × 0.25-mm internal diameter). The position of the OH groups was confirmed by the presence of the ions at m/z 259 and 345 (α cleavage at C9) and of the peaks at m/z 173 and 431 (α cleavage at C15).
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HPODE (1.3 mg/ml in 0.2M buffer of choice (0.2M potassium Pi, pH 6.8, in most other buffers showed differences in detail similar to those seen in potassium Pi, pH 6.8) were oxidized by addition of one equivalent of previously prepared 13-HPODE, precipitated immediately by adding saturated ammonium sulfate to produce 65% saturation, and brought up to 9.0. EPR spectra were examined for at least two separately prepared samples before dialysis. Lineshapes of samples before dialysis were variable because of the presence of residual ammonium sulfate. Samples were also examined in 0.2 m Tris, pH 7.5, and in 0.2 m sodium bicarbonate, pH 9.0. EPR spectra were examined for at least two separately prepared samples of each mutant. No differences in EPR spectra were found with or without the His tag present, or when the polymorphism Ser160 and Glu160 were made, and these experiments are indicated in the text. Optimal final conditions for expression were incubation at 14 °C for 30 h. The expressed proteins were purified using a nickel affinity column followed by anion exchange chromatography and a final step of high resolution gel filtration. Yields after purification were typically 10 mg of lipoxygenase/liter original culture.

**Sterespecificity of Hydrogen Abstraction from C-11 of Linoleic Acid**—Wild-type soybean LOX-1 produces almost pure 13S-HPODE from linoleic acid, and the Ala542 → Gly mutation changed this product profile to a 1:2.5:1 mixture of 13S-HPODE and 9R-HPODE (Fig. 1). The 13S and 9R positions on linoleic acid are at opposite ends of the double bond system, on the same side of the substrate molecule. The corresponding substitution of Ala542 with Ser had little effect on the reaction profile, whereas conversion to either Val or Thr resulted in complete loss of stereospecificity. As a well established feature of lipoxygenase catalysis is the initial stereoselective hydrogen abstraction (16, 18), it was of great mechanistic interest to compare this reaction in formation of both the 13S- and 9R-hydroperoxy products by the Ala542 → Gly mutant of soybean LOX-1. Accordingly, wild-type and Ala542 → Gly soybean LOX-1 were reacted separately with 11S-3H- and 11R-3H-labeled linoleic acid substrates. The 3H-labeled linoleic acids were mixed with [1-13C]linole-
leic acid as internal standard, allowing measurement of the $^3$H/$^{14}$C ratio as an index of the $^3$H content of substrate and product. The HPODE products were reduced to HODEs, converted to the methyl esters, and purified by SP-HPLC. Each sample was then run on chiral phase HPLC using the highly resolving Chiralpak AD column (26), the fractions were collected across the chromatographic peaks, and the $^3$H/$^{14}$C ratios were quantified by scintillation counting.

Incubation of wild-type soybean LOX-1 with $[11^S-3^H]$linoleic acid substrate resulted in formation of $13^S$-HPODE with loss of the $^3$H label (Fig. 2A). The $13^S$-HPODE and the $9^R$-HPODE formed by the Ala$^{542}$→Gly mutant also lost the $^3$H label (Fig. 2B and C). In contrast, when $[11^R-3^H]$linoleic acid was used as a substrate, the $^3$H label was retained in the $13^S$-HPODE formed by the wild-type soybean LOX-1 (Fig. 2D) as well as in the $13^S$-HPODE and the $9^R$-HPODE formed from the Ala$^{542}$→Gly mutant (Fig. 2E and F). All results are summarized in TABLE ONE. Clearly, all products formed from the $[11^S-3^H]$LA lost the tritium label (Fig. 3A), whereas the label was almost completely retained in products formed from the $[11^R-3^H]$LA (Fig. 3B). These results establish that the reaction to form both $13^S$-HPODE and $9^R$-HPODE by the Ala$^{542}$→Gly mutant and $13^S$-HPODE by the wild-type enzyme proceeds via the same 11-pro-$S$ hydrogen abstraction. Interestingly using linoleic acid substrate, cyclooxygenase behaves similarly to the Ala$^{542}$→Gly mutant by producing a mixture of $13^S$-HPODE and $9^R$-HPODE via pro-$S$ hydrogen abstraction (30).

**Investigation of Substrate Orientation Using C$^{16}$/LA-PC Substrate**—In view of the recent study that concluded LA and AA bind carboxyl end first in soybean LOX-1 (19), we considered it important to test substrate orientation in wild-type soybean LOX-1 and its Ala$^{542}$→Gly mutant. Given the bulk of the structure of PC and other mechanistic considerations, it is likely that the oxygenation of fatty acids esterified in PC can occur only with a tail-first entry of the substrate into the soybean LOX-1 active site. We used this criterion to investigate the substrate orientation associated with the formation of $S$ and $R$ configuration products by soybean LOX-1. 1-Palmitoyl-2-linoleoylphosphatidylcholine (C$^{16}$/LA-PC) was incubated with the wild-type and mutant enzymes, and the reduced products were transesterified to give the HODE methyl esters, which were analyzed by SP-HPLC and chiral HPLC. Wild-type soybean LOX-1 converted C$^{16}$/LA-PC to a single main product identified by SP-HPLC (after reduction and transesterification) as $13$-HODE and $9$-HODE methyl esters, respectively (Fig. 4B). Chiral phase HPLC analysis showed that the $13$-HODE metabolite had the $S$ stereoconfiguration, whereas the $9$-HODE had the $R$ stereoconfiguration (Fig. 4D and E). We confirmed also that the arachidonate ester, C$^{16}$/AA-PC, is oxygenated to the same products as
AA free acid. These experiments indicate that the change in stereospecificity caused by the Ala$^{542} \rightarrow$ Gly mutation is not the result of a change in the tail-first substrate binding orientation in the soybean LOX-1 active site.

Single and Double Dioxygenation of Arachidonic Acid—Similarly to the reactions with linoleic acid, wild-type soybean LOX-1 converted [1-$^{14}$C]AA to 15$^{S}$-HPETE as the sole product, whereas the Ala$^{542} \rightarrow$ Gly mutant converted [1-$^{14}$C]AA to 15$^{S}$-HPETE and 11$^{R}$-HPETE in a 2:1 ratio (data not shown). Other mutations at the 542 position also gave comparable results to those seen with linoleic acid (data not shown). To investigate the effect of the Ala$^{542} \rightarrow$ Gly mutation on the well characterized reaction of double dioxygenation catalyzed by soybean LOX-1 (31, 32), both the wild type and the mutant enzyme were incubated with 15$^{S}$-HPETE, and the reaction products were analyzed by RP-HPLC. As reported, wild-type soybean LOX-1 converted 15$^{S}$-HPETE to two main metabolites; the earlier-eluting peak exhibited a conjugated triene chromophore with $\lambda_{\text{max}}$ at 269 nm, characteristic of 8,15$^{S}$-diHPETE, and the second peak had a distinctive chromophore with $\lambda_{\text{max}}$ at 245 nm, characteristic of 5,15$^{S}$-diHPETE (Fig. 5A) (32). The Ala$^{542} \rightarrow$ Gly mutant incubated with 15$^{S}$-HPETE gave a different HPLC profile compared with the wild-type enzyme. The two major products were the same, but their ratio was changed in favor of the dioxygenation product at C-8. In addition, a minor but mechanistically significant new product was detectable (Fig. 5B). This product exhibited a distinctive conjugated diene chromophore with uncommonly prominent shoulders and a $\lambda_{\text{max}}$ at 239 nm (Fig. 6, inset). It was reasoned that this additional metabolite could be the “partner” of the C5 oxygenation, i.e. 9,15$^{S}$-diHPETE. To test this hypothesis, standards of 9$^{R}$-HPETE and 9$^{S}$-HPETE were prepared and reacted separately with soybean LOX-1, giving 9$^{R}$,15$^{S}$-diHPETE and 9,15$^{S}$-diHPETE products that were characterized by HPLC, UV spectroscopy, GC-MS, and $^{1}$H-NMR (“Experimental Procedures”). Each diastereomer exhibited a UV spectrum identical to the distinctive

| Table One |
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| **Measurement of $^3$H retention in experiments with stereospecifically labeled linoleic acids** |
| Product, enzyme | $^3$H retained [11$^{S}$-$^3$H]LA | $^3$H retained [11$^{R}$-$^3$H]LA |
| 13S-HODE, wild-type | 1.6 | 99.7 |
| 13S-HODE, Ala$^{542} \rightarrow$ Gly | 3.6 | 98.7 |
| 9R-HODE, Ala$^{542} \rightarrow$ Gly | 1.6 | 97.8 |

**FIGURE 3.** Hydrogen abstraction in the transformation of [11-$^{3}$H]linoleic acids by wild-type and the Ala$^{542} \rightarrow$ Gly mutant soybean LOX-1.

**FIGURE 4.** Metabolism of C$\text{_{16}}$/LA-phosphatidylcholine substrate by wild-type and mutant soybean LOX-1. A, SP-HPLC analysis of wild-type products. B, SP-HPLC analysis of Ala$^{542} \rightarrow$ Gly mutant products. C, chiral HPLC, 13-HODE-Me from wild-type. D, chiral HPLC, 13-HODE-Me from Ala$^{542} \rightarrow$ Gly mutant. Products from the incubation of C$\text{_{16}}$/AA-PC with wild-type and Ala$^{542} \rightarrow$ Gly soybean LOX-1 were reduced with TPP, converted to methyl esters by transesterification, and analyzed on a Whatman® Partisil 5 silica column (0.46 $\times$ 25 cm) eluted with hexane/isopropanol/acetic acid (100/1/0.1 by volume) at 1 ml/min with UV detection at 235 nm. Chiral HPLC analysis of the HODE methyl esters was performed as described in Fig. 1.
239 nm chromophore of the novel oxygenation product. Comparison of HPLC retention times showed that the 9\(R\), 15\(S\) standard eluted ahead of the 9\(S\), 15\(S\) diastereomers (8.4 and 9.5 min, respectively), and that the latter co-chromatographed precisely with the novel product. Accordingly, the additional metabolite of the mutant soybean LOX-1 enzyme was identified as 9\(R\), 15\(S\)-diHPETE. The 5\(S\) and 9\(R\) positions are on the same side of the fatty acid chain at opposite ends of the reacting pentadiene, and therefore 9\(R\) is the counterpart to the 5\(S\) dioxygenation product from 15\(S\)-HPETE. Notably, the relative abundance of this 9\(R\) product compared with the 5\(S\) dioxygenation product was far lower than the ratio of the 11\(R\) to 15\(S\) products found with arachidonic acid as substrate. Furthermore, no \(R\) counterpart to the 8\(S\) dioxygenation product was found. We examined the RP-HPLC traces carefully for this potential “partner” of 8\(S\), 15\(S\)-diHPETE (predicted to be a diastereomer of 14,15-diHPETE), but none was detectable. All evidence suggests that this second oxygenation involves a reversal of substrate orientation, with the carboxyl end buried deep in the active site (33). This experiment indicates that the less preferred “head first” mode of substrate binding is possible in the Ala542\(\rightarrow\)Gly mutant and that one novel \(R\) configuration product, 9\(R\), 15\(S\)-diHPETE, is formed in the process. Notably, the relative abundance of this 9\(R\) product compared with the 5\(S\) dioxygenation product was far lower than the ratio of the 11\(R\) to 15\(S\) products found with arachidonic acid as substrate. Furthermore, no \(R\) counterpart to the 8\(S\) dioxygenation product was found. We examined the RP-HPLC traces carefully for this potential “partner” of 8\(S\), 15\(S\)-diHPETE (predicted to be a diastereomer of 14,15-diHPETE), but none was detectable. All evidence suggests that this second oxygenation involves a reversal of substrate orientation, with the carboxyl end buried deep in the active site (33). This experiment indicates that the less preferred “head first” mode of substrate binding is possible in the Ala542\(\rightarrow\)Gly mutant and that one novel \(R\) configuration product, 9\(R\), 15\(S\)-diHPETE, is formed in the process. According to this model (Fig. 6), it is likely that the counterpart of the 8\(S\) dioxygenation product is not formed, because there is extended conjugation in the reacting double bond system, and the oxygenation should occur at C14, which is four carbons distant from the C10 hydrogen abstraction and out toward the exterior of the active site (Fig. 6).

**Enzyme Kinetics**—In addition to the change in product profile, the Ala542\(\rightarrow\)Gly mutation induced a significant change in reaction kinetics compared with the wild-type enzyme, Fig. 7. The maximal rates of reaction calculated as a \(k_{\text{cat}}\) of 129 s\(^{-1}\) for the wild-type and 20 s\(^{-1}\) for the mutant (not corrected for iron content, see below). The respective \(K_m\) values were calculated as 16.7 and 6.4 \(\mu\)M. The curve for the Ala542\(\rightarrow\)Gly mutant had a tendency to curve downwards at higher concentrations of linoleate, and this substrate inhibition undoubtedly confounds a meaningful estimate of \(K_m\), which instead calculates as an artificially low value. It was apparent also that the Ala542\(\rightarrow\)Gly mutant reacted with a more pronounced lag phase than evident with the wild-type enzyme (Fig. 7C). The lag could be eliminated by including a low concentration of 13\(S\)-HPODE (1.3 \(\mu\)M) in the reaction mixture, but this was effective only at low substrate concentrations (2–20 \(\mu\)M), and at higher substrate concentrations the lag reappeared. The exaggerated lag phase with the Ala542\(\rightarrow\)Gly mutant enzyme can be explained partly by its lower catalytic activity, resulting in a slower generation of sufficient hydroperoxide activator, and lower hydroperoxide/substrate ratios, both of which will accentuate the lag (34). We found also that the product specific to the Ala542\(\rightarrow\)Gly mutant, 9\(R\)-HPODE, is a very inefficient activator of the soybean LOX-1 (wild-type or mutant) compared with 13\(S\)-HPODE (Fig. 7D).

**Metal Analysis and EPR Spectroscopy**—Expressed, active LOX proteins incorporated iron at a ratio of iron/protein of 0.62, the content of inactive mutants (Thr and Val) was the same or higher, and the iron content of enzyme isolated from soybeans, and prepared for analysis
similarly, was 0.74. Therefore it can be concluded that the reduced activity of 542 mutants is not due to lack of incorporation of iron. Even though inactive mutants incorporated iron, it was possible that they had an altered iron environment, so the activated ferric state of the proteins was examined by EPR spectroscopy. All samples, wild-type and the Ala542→Gly, Ala542→Ser, Ala542→Thr, and Ala542→Val mutant enzymes, had no ferric iron EPR signals in the form originally isolated and thus were presumed to contain ferrous iron, as expected. Oxidation to ferric iron was achieved for each using one equivalent of 13S-HPODE; thus all had redox-active iron. Because EPR spectra of frozen ferric soybean LOX-1 vary depending on small molecule components of the buffer (35), care was taken to examine samples prepared under identical conditions, of similar concentrations and after thorough dialysis. The spectra shown in Fig. 8 are similar in the sense that there are three prominent peaks in each one, at g' values of ~7.27, 6.27, and 5.80 (g' value scale shown at top of Fig. 8). (For reference, if the iron symmetry showed a large change, peaks might be found any place in the magnetic field region shown in Fig. 8.) Most noticeably, the glycine mutant has diminished intensity in the peak labeled ‘1,’ possibly reflecting a change in the 5- or 6-coordinate iron liganding (see “Discussion”). Although all the other EPR spectra have similar intensities in the peaks labeled as components ‘1’ and ‘2,’ there is a broad feature, as yet uncharacterized, underlying the peaks in the EPR spectra of serine and threonine mutants that was unchanged after repurification of the samples. The valine mutant was not characterized in detail except to establish that the iron could be oxidized.

**DISCUSSION**

The results of our study are relevant to the issues of substrate access, substrate orientation, and control of oxygenation in soybean LOX-1. We have established that the initial hydrogen abstraction occurs similarly in the wild-type and Ala542→Gly mutant enzymes, with important connotations regarding substrate orientation. Furthermore, our results with single or double oxygenation reactions fit in a consistent picture of tail-first substrate binding in the initial oxygenation and reversed binding (carboxyl end first) in forming the double dioxygenation products of arachidonic acid. In considering how these results all intertwine, it is useful to understand where the Ala542 resides in the structure of soybean LOX-1. The Ala residue is located in helix 11, opposite to the iron, intruding into the available substrate binding pocket and roughly pointing “inwards” back toward the nonheme iron, which resides in the center of the catalytic domain (Fig. 9).

**Substrate Access**—Because there are no x-ray structures available for a LOX with bound fatty acid substrate (a product-bound structure is available (38)), there has always been debate on the route of substrate access to the soybean LOX-1 catalytic domain and on the mode of substrate binding (12, 15). It appears significant to us that Ala542 is
situated next to a proposed route for substrate entry (Fig. 9). In fact there is no open route in the x-ray structures of soybean LOX-1, but as noted by Minor et al. One promising [route] is located at the opposite end of cavity IIa relatively close to the Fe atom. In this case, access to IIa is barred 8–10 Å from the Fe atom by two contacts between side chain atoms: Thr259O1 and Leu541C, 4.6 Å; Lys690 and Leu541C2, 5.3 Å. StERIC unhindered adjustments of the side chains of Thr259 and Leu541 appear to be sufficient to open a channel wide enough (>6 Å) for entry of a fatty acid (15). Ala542, the determinant of oxygenase specificity, sits close to this point of access (Fig. 9), and taken together with the inferences on substrate orientation, our results are consistent with this being the point of substrate access. This also matches the mode of substrate access and substrate binding inferred from the x-ray structure of the mammalian reticulocyte 15-LOX (which was crystallized with an inhibitor in the active site) (20, 39), suggesting many common features in the respective reactions. It might be noted that helices 2 and 21 have side chains in contact with helix 11 and other entries into cavity IIa involving these three helices can also be considered (15).

**Substrate Orientation**—There remain conflicting views on how substrates bind in different lipoxgenases (e.g., Refs. 21, 33, and 40–42). A recent study of soybean LOX-1, the enzyme we studied here, used the results of mutagenesis of two internal amino acids (Trp500 and Arg707) to conclude that the usual orientation of arachidonic acid (as it is converted to 15S-HPTE) is with its carboxyl end buried deep in the protein (19). But this conclusion fails to consider the well established fact that linoleate or arachidonate esterified in phosphatidylcholine are oxygenated by soybean LOX-1 identically to the free fatty acids (43). Here we show that this applies also to reaction of C16/LA-PC and C16/AA-PC with the Ala542 Gly mutant, which forms the 9R/15S linoleate products or 11R/15S arachidonate products from the PC ester or the free acids. This is only compatible with a tail-first substrate binding orientation in the soybean LOX-1 active site in all these primary oxygenation reactions. Tail-first binding is further supported by the experiments with stereospecifically labeled linoleic acids showing that the same hydrogen is abstracted to form products with either S or R specificity. Also consistent with the conclusion is the positioning of the Ala542 residue (or Gly542 in the mutant) in the active site, closer to the surface of the enzyme compared with the iron, allowing control of oxygenation at C-9 of linoleate when the fatty acid is bound in the tail-first orientation. Binding in the reversed orientation with the carboxyl end deep in the active site is implicated in some circumstances, notably in the further oxygenation of 15S-HPETE. Here we have provided additional consistent support for this in characterizing oxygenation of 15S-HPETE to 9R,15S-diHPETE by the Ala542 Gly mutant enzyme, thus identifying the R configuration “partner” of the usual secondary oxygenation product 5S,15S-diHPETE. These second oxygenation reactions are consistent with substrate binding in the reversed carboxyl end-first orientation.

Our results, therefore, are consistent with the positioning of C9 of linoleic acid in the vicinity of Ala542, and computational docking of the
substrate in the active site gave several solutions matching this arrange-
ment (Fig. 9). Many conformations of linoleic acid can be achieved 
inside the active site because of the high flexibility of the substrate, and 
several mechanistically relevant possibilities are illustrated in the figure.
Linoleic acid is shown with the middle of the carbon chain lying beside 
the iron and with its methyl end running deep into the active site. The 
location of Ala542 opposite to the iron is favorable for interference with 
antarafacial oxygenation on C-9. As discussed above, we hypothesize 
that the linoleic acid enters the active site through the channel opened 
by movement of the side chains of Thr259 and the Leu541 residues, but 
the docking program cannot accurately account for this conformational 
flexibility of the protein. As a consequence, the linoleic acid appears to 
be trapped in the active site with its carboxylic end computed to be 
turning back inwards, rather than exiting the active site.

**Oxygen Access to the Activated Radical—**Access of oxygen to the 
pentadienyl radical intermediate of LOX appears to be altered in the 
Ala542 → Gly mutant. It has been argued that oxygen approaches the 
radical intermediate through a side channel near Ile553 and that this 
channel controls 13S oxygenation reactions of the wild type enzyme 
(14). Here we observe the appearance of 9R oxygenation in the Ala542 → 
Gly mutant in which a methyl group has been removed at position 542.
According to our analysis it is unlikely that this could create a new 
specific channel for 9R oxygenation. And if 9R oxygenation can occur 
without the need for an oxygen channel, this raises the question whether 
a channel is the basis of stereocountrol in the usual 13S reaction. On 
the other hand, it is quite probable that an oxygen pocket (a space to “hold” 
an O₂ molecule next to the substrate) forms part of the structural basis 
of stereocountrol. This is compatible with the effects of changing Ala542 
to Gly and introducing the new 9R oxygenase activity on the carboxyl-
proximal end of the linoleate molecule. In concert with this, steric fac-
tors will shield the reacting pentadiene and thus control positional and 
stero specificity. Thus, the most straightforward interpretation of the 
effects of the Ala542 → Gly mutation is that it opens up space and allows 
oxygenation on the side of the activated pentadienyl radical closer to the 
surface of the enzyme.

**Other Changes in the Active Site—**EPR spectroscopy and the reaction 
kinetics suggest that the Ala542 mutations are also associated with some 
changes in the way the substrate can interact with the catalytic iron 
center. General access of fatty acids is clearly not blocked in the inactive 
mutants, because hydroperoxide product oxidizes iron in all Ala542 
mutants examined. And because the serine and threonine mutants are 
active and inactive, respectively, and have similar EPR spectra to wild-
type, the data suggest that the inactivity of the threonine mutant results 
from changes in substrate positioning relative to the catalytic iron rather 
than from a major change in the properties of the iron center.
The EPR spectrum (Fig. 8A) of the glycine mutant is similar to the 
wild type spectrum in that there are two major components corre-
sponding to the same degrees of deviation from axial symmetry at iron, 
but there is considerable variation in their proportions. The signals in 
the EPR spectra and their intensity are a reflection of the detailed inter-
actions of iron and its ligands, and the two signals may correspond to 5- 
and 6-coordinate iron liganding. Previously, a network of H-bonds 
involving iron ligand Asn594 and residues in the iron second coordina-
tion sphere has been characterized by mutation, CD, magnetic circular 
dichroism, and x-ray structure analyses, with the conclusion that 
dynamic changes in iron coordination, and especially in the iron-Asn594 
bond length, is essential for catalytic function (44, 45). It is likely that 
the helix 11 containing Ala or Gly at 542 is connected through hydrogen 
 bonds of residues and waters in cavity Ila to the network of H-bonds 
that modulate the iron-ligand bond lengths. We speculate that the vol-
ume of the side chain at position 542 in soybean LOX-1 influences the 
hydrogen bonding details and/or the location of waters throughout cav-
ity Ila. Explaining precisely how these networks are linked awaits char-
acterization of the structure of relevant enzyme intermediates in lipoxy-
genase catalysis. In addition, the dramatic effects of the Ala-Gly 
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in the attainable Vmax(kcat) of the enzyme. This may indicate effects 
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