Biosynthesis, isolation, and NMR analysis of leukotriene A epoxides: substrate chirality as a determinant of the *cis* or *trans* epoxide configuration[S]

Jing Jin, Yuxiang Zheng,[1] William E. Boeglin, and Alan R. Brash[2]

Department of Pharmacology and the Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, TN 37232

Abstract **Leukotriene (LT)A_4 and closely related allylic epoxides are pivotal intermediates in lipoxygenase (LOX) pathways to bioactive lipid mediators that include the leukotrienes, lipoxins, eoxins, resolvins, and protectins. Although the structure and stereochemistry of the 5-LOX product LTA_4 is established through comparison to synthetic standards, this is the exception, and none of these highly unstable epoxides has been analyzed in detail from enzymatic synthesis. Understanding of the mechanistic basis of the *cis* or *trans* epoxide configuration is also limited. To address these issues, we developed methods involving biphasic reaction conditions for the LOX-catalyzed synthesis of LTA epoxides in quantities sufficient for NMR analysis. As proof of concept, human 15-LOX-1 was shown to convert 15*S*-hydroperoxy-eicosatetraenoic acid (15*S*-HPETE) to the LTA analog 14*S*,15*S*-*trans*-epoxy-eicosa-5*Z*,8*Z*,10*E*,12*E*-tetraenoate, confirming the proposed structure of eoxin A4 Using this methodology we then showed that recombinant *Arabidopsis* AtLOX1, an arachidonate 5-LOX, converts 5*S*-HPETE to the *trans* epoxide LTA_4 and converts 5*R*-HPETE to the *cis* epoxide 5-*epi*-LTA_4, establishing substrate chirality as a determinant of the *cis* or *trans* epoxide configuration. The results are reconciled with a mechanism based on a dual role of the LOX nonheme iron in LTA epoxide biosynthesis, providing a rational basis for understanding the stereochemistry of LTA epoxide intermediates in LOX-catalyzed transformations.—** Jin, J., Y. Zheng, W. E. Boeglin, and A. R. Brash. **Biosynthesis, isolation, and NMR analysis of leukotriene A epoxides: substrate chirality as a determinant of the *cis* or *trans* epoxide configuration. *J. Lipid Res.* 2013.** 54: **754–761.**

Supplementary key words 5-lipoxygenase • 15-lipoxygenase • hydroperoxy-eicosatetraenoic acid • nuclear magnetic resonance

The leukotrienes (LTs) are a family of lipid mediators derived from arachidonic acid and implicated in the pathogenesis of asthma and other inflammatory diseases (1). The biosynthetic pathway, unraveled three decades ago, is a model for related transformations to anti-inflammatory mediators including the lipoxins, eoxins, resolvins, protectins, and maresins (2–5). 5-Lipoxygenase (5-LOX) catalyzes the first two steps in leukotriene biosynthesis, namely the stereospecific oxygenation of arachidonic acid to 5*S*-hydroperoxy-eicosatetraenoic acid (5*S*-HPETE), and dehydration of the hydroperoxide to LTA_4, the allylic 5,6-*trans*-epoxide from which the bioactive leukotrienes are derived (**Fig. 1**) (6–9). The importance of this pivotal intermediate is reflected in the extensive efforts directed toward perfecting the total synthesis of LTA_4 and of closely related 5,6-epoxide isomers (as reviewed in Refs. 10–12).

LTA_4 is highly unstable in physiological buffer at pH 7.4, with a half-life estimated as approximately 3 s at 25°C and 18 s at 4°C (13). Despite this instability, LTA_4 was detected as an evanescent intermediate in short-term incubations of arachidonic acid with human leukocytes (6), and the radiolabeled product was isolated and, as the methyl ester derivative, shown to have similar chromatographic mobility and hydrolysis under acidic conditions as a synthetic standard (14). The stereochemistry has heretofore not been established directly on the biosynthetic product but was deduced from an understanding of the biosynthetic pathway and by study of the reactions of synthetic LTA_4 and related isomers in transformations to the stable, bioactive leukotrienes LTB_4 and LTC_4 (15–17). Herein we report the isolation and purification of LTA epoxides as products of LOX enzymes in quantities sufficient for NMR analysis of the stereoconfigurations. The isolation method

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Abbreviations: HPETE, hydro(pero)xyeicosatetraenoic acidLT, leukotriene; LOX, lipoxygenase; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; RP-HPLC, reverse phase high-pressure liquid chromatography; SP-HPLC, straight phase high-pressure liquid chromatography; UV, ultraviolet.

[1] Present address: Department of Systems Biology, Harvard University, Boston, MA 02115

[2] To whom correspondence should be addressed.
e-mail: alan.brash@vanderbilt.edu

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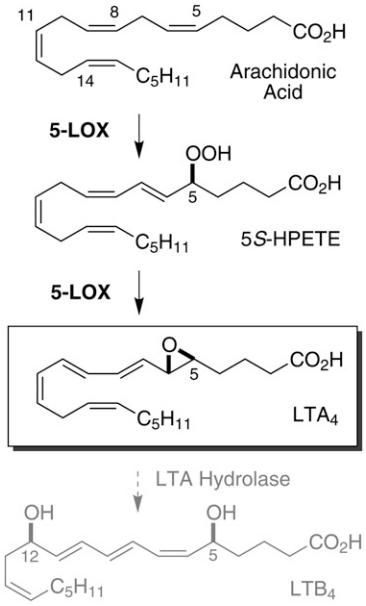

Fig. 1. The 5-LOX catalyzed pathway of LTA_4 biosynthesis. 5-LOX oxygenates arachidonic acid to 5*S*-HPETE and then dehydrates the hydroperoxide to LTA_4. Shown in gray, metabolism by LTA_4 hydrolase produces the bioactive leukotriene LTB_4.

we developed here was successfully applied earlier to allene oxides (18) and to analysis of an LTA-related epoxide synthesized by a catalase-related hemoprotein (19), although not heretofore applied to lipoxygenase reactions. We identify substrate chirality as a determinant of the *cis* or *trans* configuration of the LTA epoxide and propose a model that predicts how these products are derived in the enzymatic transformation.

MATERIALS AND METHODS

Materials

Arachidonic acid and its methyl ester were purchased from NuChek Prep Inc. (Elysian, MN). Soybean LOX-1 (lipoxidase, type V) and α-tocopherol were purchased from Sigma (St. Louis, MO). 15*S*-HPETE was synthesized by reacting soybean LOX-1 with arachidonic acid followed by straight-phase HPLC (SP-HPLC) purification.

Synthesis and purification of enantiomeric HPETEs

Racemic hydroperoxides were prepared by vitamin E-controlled autoxidation (20). Arachidonic acid methyl ester (500 mg) was transferred to a 2 liter round-bottomed flask, mixed with 10% (w/w) α-tocopherol, and evaporated to dryness. The flask filled with oxygen, capped, and placed in an oven at 37°C. The oxygen was replenished daily. After 3 days, the lipid was dissolved in 10 ml of methylene chloride and stored at −30°C. The autoxidation sample was fractionated and partly purified using a 5 g silica Bond-Elut (Varian, Palo Alto, CA) with the solvents of hexane/ethyl acetate (three fractions of 10 ml of 95:5, followed by three of 10 ml of 90:10 and three of 10 ml of 85:5, v/v); the HPETE methyl esters were eluted in fractions 6 through 9. Racemic 5-HPETE and 15-HPETE methyl esters were then separated from other positional HPETE isomers by SP-HPLC using a semi-preparative Ultrasphere 10 μm silica column (25 × 1 cm) with a solvent system of hexane/isopropanol (100/1, by volume) run at a flow rate of 4 ml/min (Beckman) . The enantiomeric HPETE methyl esters were resolved using a semi-preparative, 25 × 1 cm Chiralpak AD column (Chiral Technologies Inc., West Chester, PA) with a solvent system of hexane/methanol (100:2, by volume) run at a flow rate of 4 ml/min (21).

To prepare the corresponding free acids, the purified HPETE methyl esters (1 mg) in 2 ml methanol/methylene chloride (10:1, by volume) were brought to room temperature, and an equal volume of 1 M KOH was added. The sample was mixed and kept at room temperature under an atmosphere of argon with occasional sonication in a water bath. After 20 min, the sample was acidified to pH 4.5 and extracted with an equal volume of methylene chloride. The organic phase was washed twice with water and then evaporated to dryness under a stream of nitrogen. The dried sample was redissolved in methanol and stored at −30°C. HPETEs were quantified based on the conjugated diene chromophore at 236/237 nm, ε = 25,000 (10 μg/ml = 0.75 AU).

Expression and purification of human 15-LOX-1 and *Arabidopsis thaliana* AtLOX1

The cDNA of human 15-LOX-1 was subcloned into the pET3a vector (with an N-terminal His6 tag), and the protein was expressed in BL21 cells. A typical preparation of a 100-ml culture was carried out as follows: 100 ml of 2XYT medium containing 100 μg/ml ampicillin was inoculated with a single colony of h15-LOX-1-His in BL21 cells and grown at 37°C at 250 rpm until OD600 reached 0.8. Isopropyl β-D-1-thiogalactopyranoside (0.5 mM) was then added to the culture, which was grown at 16°C, 220 rpm for 4 days. The cells were spun at 5,000 *g* for 20 min in a Beckman Avanti J-25I centrifuge, washed with 40 ml of 50 mM Tris (pH 7.9), pelleted again at 5,000 *g* for 20 min, and resuspended in 10 ml of 50 mM Tris (pH 8.0), 500 mM NaCl, 20% glycerol, and 100 μM PMSF. The spheroplasts were sonicated five times for 10 s using a model 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) at a setting of 5. CHAPS detergent was added at a final concentration of 1% (w/w), and the sample was kept on ice for 20 min. The resulting membranes were spun at 5,000 *g* for 20 min at 4°C. h15-LOX-1 activity was present in the supernatant. The supernatant was loaded on a nickel-NTA column (0.5 ml bed volume; Qiagen, Gaithersburg, MD) equilibrated with 50 mM Tris buffer (pH 8.0), 500 mM NaCl. The column was then washed with the equilibration, buffer and the nonspecific bound proteins were eluted with 50 mM Tris buffer (pH 8.0), 500 mM NaCl, and 50 mM imidazole. The h15-LOX-1 was then eluted with 50 mM Tris buffer (pH 8.0), 500 mM NaCl, and 250 mM imidazole. Fractions of 0.5 ml were collected and assayed for the LOX activity. The positive fractions were dialyzed against 50 mM Tris buffer (pH 7.5) and 150 mM NaCl. The purity of the enzyme preparations was determined by SDS-PAGE and Coomassie Blue staining; the prominent band of h15-LOX-1 accounted for about 80% of the total protein.

The cDNA of *Arabidopsis thaliana* AtLOX1 was subcloned into the pET14b vector (with an N-terminal His6 tag). The protein was expressed in *Escherichia coli* BL21 (DE3) cells and purified by nickel affinity chromatography according to a previously published protocol (22).

Biphasic reaction conditions for preparation of LTA epoxides

Enzyme reactions were performed at 0°C, with the HPETE substrate initially in hexane (5 ml, bubbled for 30 min before use with argon to decrease the O_2 concentration, and containing ~200 μM HPETE) layered over the recombinant LOX enzyme (1–2 mg; ~20 nmol) in 400 μl of Tris buffer (pH 7.5 for h15-LOX-1 and pH 6.0 for AtLOX1). The reaction was initiated by

vigorous vortex mixing of the two phases. After 1.5 min, the hexane phase was collected and scanned from 200 to 350 nm in ultraviolet (UV) light by using a Perkin-Elmer Lambda-35 spectrophotometer. The hexane phase was evaporated to about 2 ml under a stream of nitrogen, treated with ethanol (20 μl) and ethereal diazomethane for 10 s at 0°C, rapidly blown to dryness, and kept in hexane at −80°C until further analysis.

HPLC analyses

Aliquots of the methylated hexane phase were analyzed by RP-HPLC using a Waters Symmetry column (25 × 0.46 cm) using a solvent of MeOH/20 mM triethylamine (pH 8.0) (90/10 by volume) at a flow rate of 1 ml/min, with on-line UV detection (1100 series diode array detector; Agilent) (19). Further purification was achieved by SP-HPLC using a Beckmann Ultrasphere 5 μm silica column (25 × 0.46 cm) using a solvent of hexane/triethylamine (100/0.5) run at 1 ml/min.

NMR analysis

^{1}H NMR and ^{1}H,^{1}H COSY NMR spectra were recorded on a Bruker AV-III 600 MHz spectrometer at 283 K. The parts/million values are reported relative to residual nondeuterated solvent (δ = 7.16 ppm for C_6H_6). Typically, 1,024 scans were acquired for a 1-D spectrum on ∼20 μg of LTA epoxide.

GC-MS analysis of 5-oxo-ETE

The methoxime derivative was prepared by treatment with methoxylamine hydrochloride (10 mg/ml in pyridine) at room temperature for 16 h. The trimethylsilyl ester derivative was then prepared using bis(trimethylsilyl)-trifluoroacetamide/pyridine (12 μl, 5:1, v/v) for 2 h at room temperature. Analysis of the trimethylsilyl ester methoxime derivative was carried out in the positive ion electron impact mode (70 eV) using a Thermo Finnigan Trace DSQ ion trap GC-MS with the Xcalibur data system. Samples were injected at 150°C, and after 1 min the temperature was programmed to 300°C at 20°C/min. The spectrum was recorded by repetitive scanning over the range of 50–500 *m/z*.

RESULTS

Method development, preparation, and analysis of 14,15-LTA$_4$

To prepare and isolate LTA epoxides, we used a biphasic reaction system, kept at 0°C, with the HPETE substrate dissolved in hexane and a highly active LOX enzyme in aqueous buffer. Upon vigorous mixing, the more polar hydroperoxide partitions into the aqueous phase and reacts with enzyme, and the less polar epoxide product instantly back-extracts into the hexane and is thereby protected from hydrolysis. For the initial development and validation of the method, we required an enzyme capable of LTA-type epoxide synthesis and with the highest possible catalytic activity. Our preparation of recombinant human 15-LOX-1 proved suitable; it converts its primary product, 15*S*-HPETE, to several derivatives, including the LTA$_4$ analog 14,15-LTA$_4$ (23).

In optimizing the conditions for the reaction of 15*S*-HPETE with 15-LOX-1, we used a 12.5-fold excess of hexane over the aqueous phase (pH held at 7.5) and a mixing time of 90 s at 0°C. Comparison of the UV spectrum of the hexane phase before and after vortex mixing provided quick feedback on the extent of reaction. In the example shown in **Fig. 2**, a new conjugated triene chromophore typical of an LTA-type epoxide (λ_{max} 280 nm) is evident after mixing. The substrate concentration has diminished markedly and is only partially replaced by the conjugated triene. Hydrolysis of the epoxide produces 8,15- and 14,15-dihydroxy derivatives that do not extract into hexane, especially at the elevated pH of 7.5, and the same applies to any dioxygenation products or epoxyalcohol derivatives (23). Analysis of the aqueous phase from these biphasic reactions revealed the presence of dihydro(pero)xy derivatives (prominently with all-*trans* conjugated trienes, λmax 269 nm, typical of epoxide hydrolysis products, but including dihydroperoxides) as well as a mixture of more polar derivatives with *trans-trans* conjugated dienes (not identified, but possibly epoxyalcohols formed from conjugated triene-containing dihydroperoxides). Overall, about 60% of the starting 15*S*-HPETE was consumed, with an estimated recovery of LTA-type epoxide under the above reaction conditions of 10–15% (taking into account the molar absorbance of the conjugated trienes is about 1.5–2 times the value for a conjugated diene) (24, 25), with the balance of products being accounted for mainly by the nonextractable, more polar derivatives.

After preparing the methyl esters of the hexane extract by brief reaction with diazomethane at 0°C, the remaining 15*S*-HPETE and its products were analyzed on RP-HPLC and SP-HPLC using conditions suitable for LTA-type epoxides (13, 26). **Fig. 3** illustrates RP-HPLC analysis with UV detection at 270 nm. The unreacted 15*S*-HPETE (at 7.2 min retention time, the largest peak on the chromatogram but weakly detected at 270 nm) is immediately followed by a minor keto derivative (at 7.6 min retention time, a conjugated dienone, λ_{max} 281 nm) and then by a well-resolved peak of the putative LTA-type epoxide (at 9.7 min, a conjugated triene, λ_{max} 280 nm). This product was collected from SP-HPLC runs with a solvent of hexane containing 0.1% triethylamine. The pooled aliquots of LTA epoxide methyl ester (∼20 μg) were analyzed by ^{1}H-NMR in d_6-benzene (**Fig. 4**). The 2-D COSY spectrum (described

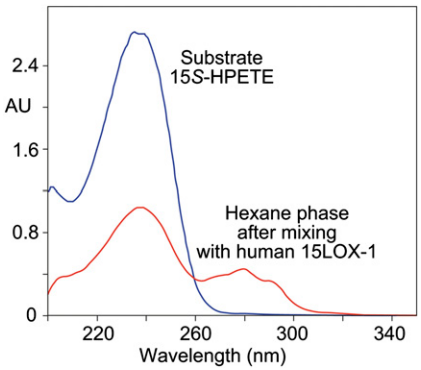

Fig. 2. UV analysis of LTA epoxide formation under biphasic reaction conditions. The UV spectrum of 15*S*-HPETE substrate (40 μg/ml) in 5 ml hexane was recorded before the reaction and after vortex mixing for 90 s at 0–4°C with 1.5 mg 15-LOX-1 enzyme in 400 μl 0.1 M Tris (pH 7.5).

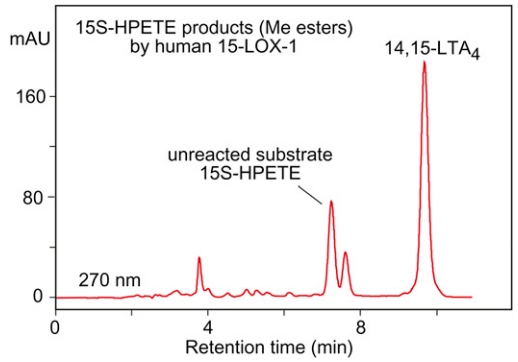

Fig. 3. RP-HPLC analysis of the reaction of 15-LOX-1 with 15*S*-HPETE. RP-HPLC analysis used a Waters Symmetry C18 column (0.46 × 25 cm), a flow rate of 1 ml/min, and a solvent system of methanol/20 mM triethylamine (pH 8.0) (90/10, by volume) with UV detection at 270 nm.

below) helps confirm the proton assignments. The expanded regions for the olefinic protons (5.0–6.5 ppm) and the epoxide protons (2.6–3.1 ppm) illustrate the splitting of individual signals and associated coupling constants from which the stereochemistry can be derived. These provide the configuration of the double bonds, in particular identifying the conjugated triene as 8*Z*,10*E*,12*E*, and on the epoxide protons the 2 Hz coupling between H14 and H15 identifies the epoxide configuration as *trans*. Based on these analyses and with the reasonable assumption that the original 15*S* configuration is retained, the structure of the epoxide product can be defined as 14*S*,15*S*-*trans*-epoxy-eicosa-5*Z*,8*Z*,10*E*,12*E*-tetraenoate. This confirms the structure originally proposed for this intermediate (23, 27), now named eoxin A_4, precursor of the proinflammatory eoxins of human eosinophils in asthma (3, 28).

Leukotriene epoxide formation from enantiomeric HPETE substrates

To investigate the significance of substrate chirality to LTA epoxide formation, initially we examined the reaction of 15-LOX-1 with the mirror image substrate, 15*R*-HPETE. The analyses showed, however, that the major product by far is the keto derivative 15-oxo-eicosatetraenoic acid (data not shown). Only traces of a mixture of very minor products exhibiting an LTA-related UV chromophore were detectable.

With the requirement that the LOX enzyme used in these preparations should have very high catalytic activity (which our available preparations of mammalian 5-LOX did not), we switched to the use of recombinant *Arabidopsis* AtLOX1 as the arachidonate 5-LOX (22). This enzyme is a homolog of the potato 5-LOX (22) that is capable of LTA epoxide synthesis and has been used as a model for the study of 5-LOX-catalyzed leukotriene synthesis in earlier studies (7, 29, 30). Although the native activity of these plant enzymes is as a linoleic acid 9*S*-lipoxygenase, they form 5*S*-HPETE as a prominent product from arachidonic acid and can further convert this to LTA_4 (22, 29, 30). Indeed, after vortex mixing of AtLOX1 with 5*S*-HPETE at 0°C, UV spectroscopy of the hexane phase showed a decrease in

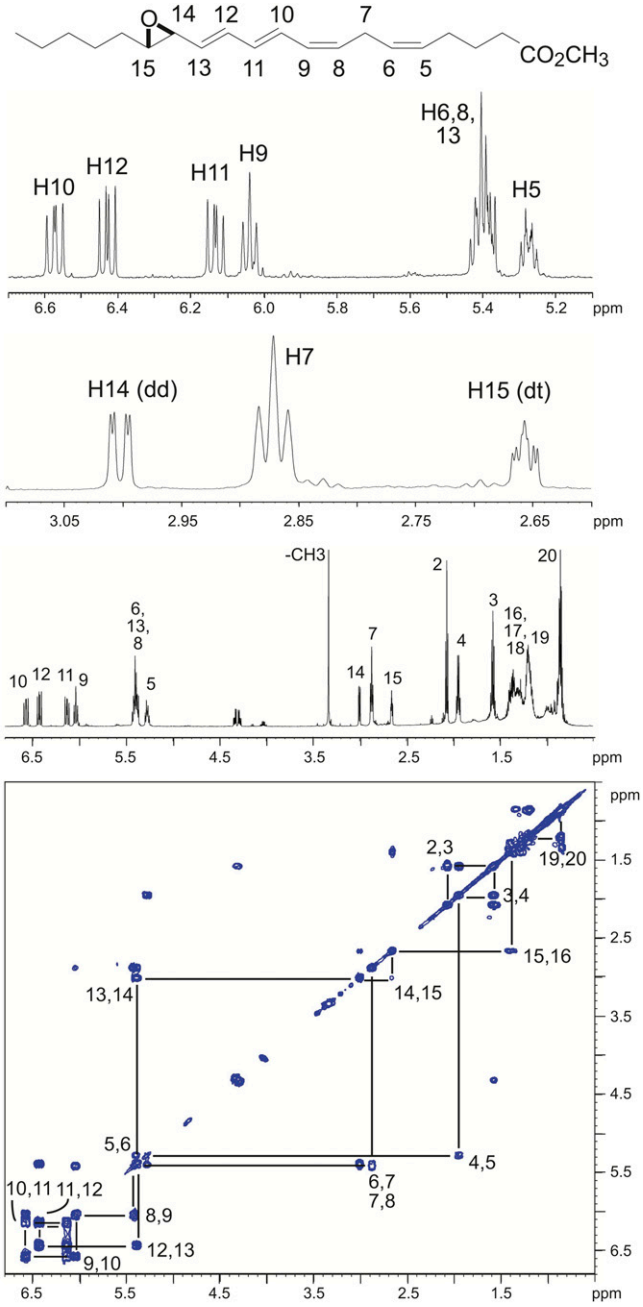

Fig. 4. NMR analysis of 14,15-LTA_4. Spectra were recorded in d6-benzene at 283 K using a Bruker 600 MHz spectrometer. The two-dimensional H,H-COSY spectrum is shown below; shown above is an expanded view of the olefinic protons (5.0–6.5 ppm) and epoxide protons (2.6–3.1 ppm).

substrate and appearance of a new chromophore with λ_{max} at 280 nm, characteristic of LTA_4 epoxide (**Fig. 5A**). After methyl esterification with diazomethane, the product was analyzed by RP-HPLC (Fig. 5B). The complete structure was then established by ^{1}H-NMR (see supplementary data). Most importantly, this confirmed the 5,6-*trans* configuration of the epoxide ($J_{5,6}$ = 2 Hz) (31) (Fig. 5C).

Incubation of the 5*R* enantiomer of 5-HPETE with 5-LOX was performed under the same conditions. UV spectroscopy of the hexane phase after vortex mixing showed the appearance of a less well-defined spectrum in the 280 nm

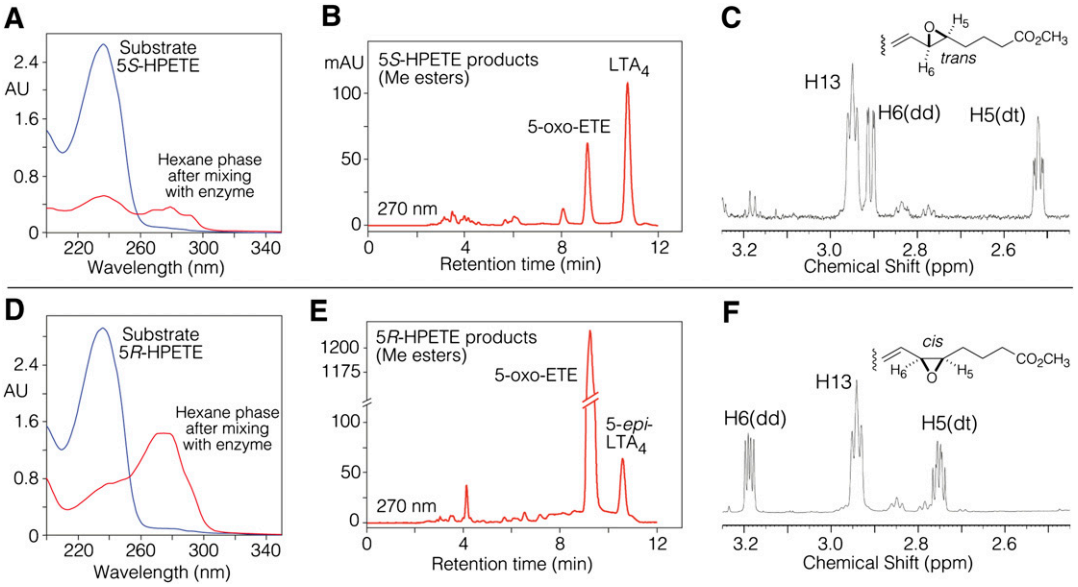

Fig. 5. Product analyses from reactions of 5*S*- and 5*R*-HPETE with 5-LOX. A: UV spectrum of 5*S*-HPETE in hexane before and after vortex mixing with 5-LOX enzyme (AtLOX1). B: Reversed-phase HPLC analyses of the product methyl esters from 5*S*-HPETE. Column: Waters Symmetry C18, 25 × 0.46 cm; solvent, methanol/20 mM triethylamine (pH 8.0) (90/10, by volume); flow rate, 1 ml/min, UV detection at 270 nm. There was no sign on our HPLC chromatograms of a conjugated triene-containing keto derivative, a reported nonenzymatic rearrangement product of LTA_4 epoxide (46). C: Partial ^{1}H-NMR spectrum (2.45–3.25 ppm) of the *trans*-LTA epoxide product from 5*S*-HPETE. *D–F*: The UV (D), HPLC (E), and NMR (F) analyses of 5*R*-HPETE reaction with 5-LOX.

region, comprised of a mixture (Fig. 5D). RP-HPLC analysis of the esterified hexane phase demonstrated two products of the incubation (Fig. 5E). The major product exhibited a dienone chromophore with λ_{max} in MeOH/H_2O (90:10) at 281 nm. Its structure was identified as 5-oxo-eicosatetraenoic acid (5-oxo-ETE) by GC-MS analysis of its trimethylsilyl ester methoxime derivative (supplementary Fig. I), and by reduction with $NaBH_4$ to 5-hydroxyeicosatetraenoic acid, identified by comparison to an authentic standard. The less abundant product displayed a conjugated triene chromophore with λ_{max} at 280 nm. The structure of this LTA epoxide derived from 5*R*-HPETE was established by ^{1}H-NMR (see supplementary data), most significantly defining the 5,6-epoxide configuration as *cis* ($J_{5,6}$ = 4 Hz), thus identifying the product as the 5*R*,6*S* *cis* epoxide, 5-*epi*-LTA_4 (Fig. 5F). Its yield was similar to that of LTA_4 (the dominant recovery of 5-oxo-ETE from 5*R*-HPETE being in excess of its production from 5*S*-HPETE). Comparison of the NMR spectra of LTA_4 and its 5-epimer also defined their identical double bond configurations as 7*E*,9*E*,11*Z*,14*Z* (**Fig. 6A**). Their UV spectra are almost superimposable, with the difference only discernible with the two overlaid (Fig. 6B).

DISCUSSION

The dual purposes of our study were to enable the direct structural analysis of the unstable LTA-type epoxides from lipoxygenase reactions and to further the understanding of factors that control their precise stereochemistry. The structure of the classic 5-lipoxygenase product, LTA_4, was established beyond doubt by comparison of transformations of the natural product and leukotriene epoxides prepared by total chemical synthesis (15–17). It has been widely assumed that other LOX-catalyzed LTA-type products will be the direct structural analogs of LTA_4, although the equivalent experimental support is lacking. The possibility that LOX reactions could lead to *cis*-epoxy LTA_4 was deduced by Corey and coworkers (32), who identified the stereochemistry of the initial LOX-catalyzed hydrogen abstraction as a determinant of the *trans* or *cis* epoxide configuration. Herein we identified the stereochemistry of the HPETE substrate as an additional determinant. In the following discussion we present a model that rationalizes these observations and predicts the LTA epoxide stereochemistry in novel LOX-catalyzed reactions.

To explain the available results, we further developed a conceptual model underlying the mechanistic basis of the LOX-catalyzed transformation of HPETE precursor to LTA_4-type epoxide (**Fig. 7**). The nonheme iron in the LOX active site must be involved in the initial hydrogen abstraction from the HPETE substrate and cleavage of the hydroperoxide moiety (33, 34). Key to our thinking, therefore, is that the hydrogen and hydroperoxide should exhibit a suprafacial relationship. Because the conversion of [10*R*-^{3}H]5-HPETE to LTA_4 is associated with a primary isotope effect resulting in an enrichment in the specific activity of the unreacted substrate (35–37), this provides critical evidence identifying the 10*pro-R* hydrogen abstraction as the first irreversible step in leukotriene A_4 biosynthesis. This implicates the ferric iron (hydroxide) in 5-LOX as the active species catalyzing the first step of the transformation. The observation that reducing inhibitors of the lipoxygenase that leave the active site iron in the ferrous state block the dioxygenase reaction and LTA synthesis (7, 23)

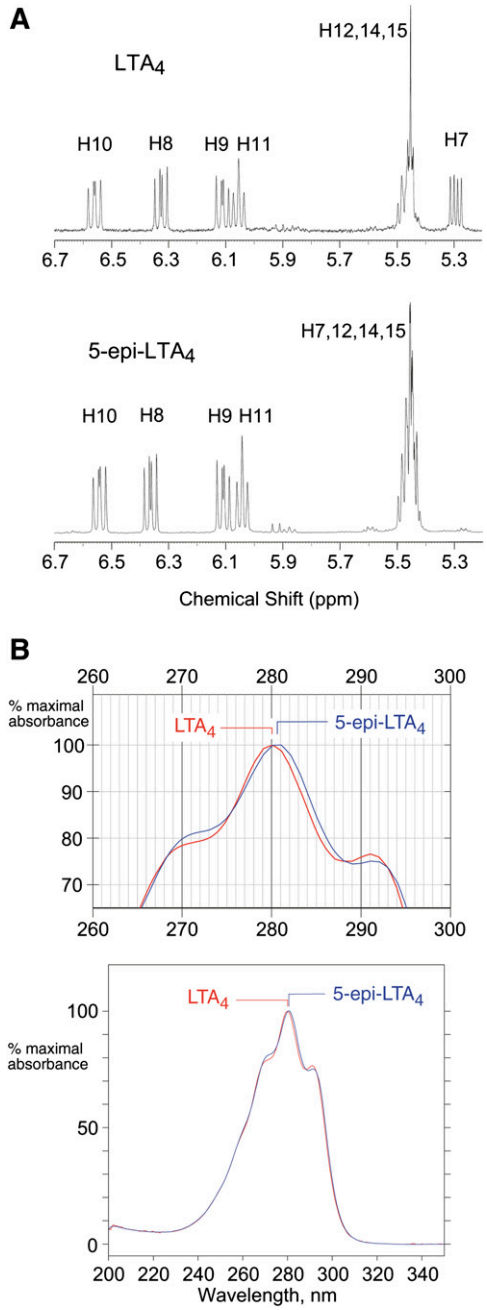

Fig. 6. Spectral comparison of LTA_4 and 5-*epi*-LTA_4. A: NMR spectra of the olefin protons in LTA_4 and 5-*epi*-LTA_4 methyl esters. B: Overlay of the UV spectra of LTA_4 and 5-*epi*-LTA_4 (*cis*-epoxy, 5*R*,6*S*). Top: detailed view. Bottom: full spectra, 200–350 nm.

provides further support for involvement of the ferric iron in catalyzing the initial hydrogen abstraction:

$$Fe^{3+}-OH + HR-OOH \rightarrow Fe^{2+}-OH_2 + \bullet ROOH$$

Further reaction will entail homolytic cleavage of the hydroperoxide, thus cycling the lipoxygenase ferrous iron back to ferric, because radical recombination produces the epoxide product:

$$Fe^{2+}-OH_2 + \bullet ROOH \rightarrow Fe^{3+}-OH + H_2O + R(O)$$

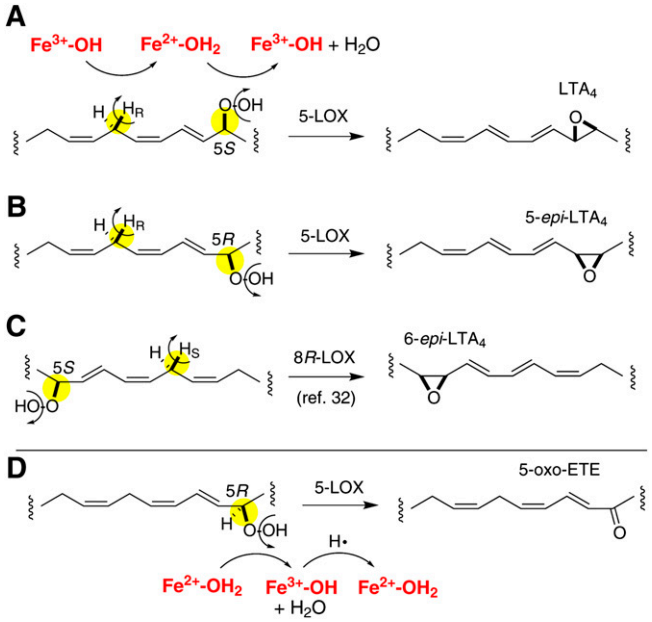

Fig. 7. Lipoxygenase-catalyzed transformations to LTA epoxides. LTA synthesis is initiated by the ferric form of the LOX enzyme (A–C), whereas dehydration to the ketone involves the ferrous enzyme (*D*, below the dividing line).

It follows from this that the iron must have access to the initial hydrogen abstracted and to the hydroperoxide (i.e., the two are on the same face of the substrate). In achieving this, 5*S*-HPETE (the natural enantiomer) must assume the *transoid* conformation at the 5-carbon, thus dictating the natural *trans*-epoxy configuration of LTA_4 (Fig. 7A). The enantiomeric substrate, 5*R*-HPETE, has to assume the *cisoid* conformation, which results in its transformation to 5-*epi*-LTA_4, now established experimentally (Fig. 7B).

To complete the picture with related transformations, Fig. 7C illustrates the result reported by Corey and coworkers (32) in which an 8*R*-LOX activity, which catalyzes *pro-S* hydrogen abstraction from C-10 (38), converted 5*S*-HPETE to the *cis* epoxide 6-*epi*-LTA_4 (32); by contrast, 5*S*-LOX, which abstracts the 10*pro-R* hydrogen (30), produced *trans*-epoxy LTA_4 from 5*S*-HPETE, as expected (32). The epoxides were identified indirectly based on a difference in pattern of the hydrolysis products in comparison to hydrolysis of the synthetic standards (32). Because it is now known that *R* and *S* lipoxygenases are closely related enzymes (39, 40) and that substrate is exposed to the same open ligand position of the active site iron (41), we deduce that the 5*S*-HPETE substrate binds in an opposite head-to-tail orientation in 5*S*-LOX and 8*R*-LOX to present the appropriate C-10 hydrogen for abstraction by the nonheme iron (42). Accordingly, the substrate is shown in the reversed orientation in the 8*R*-LOX reaction illustrated in Fig. 7C. Reactions that appear feasible "on paper" may or may not be executed favorably, depending on steric considerations within the LOX enzyme. This is evident in the reaction of 15*R*-HPETE with 15-LOX-1 in which LTA epoxide synthesis is formed in only trace amounts. Similarly, in the reaction of 5*R*-HPETE with 5-LOX, the formation of 5-*epi*-LTA_4 is superseded by 5-oxo-ETE synthesis as the

major product in catalysis initiated by the ferrous nonheme iron (Fig. 7D) (43, 44).

The concepts developed here on the determinants of *cis* or *trans* LTA_4 epoxide configuration establish a rational mechanism underlying the structures of these key biosynthetic intermediates. The concepts can be applied to the enzymatic formation of novel LTA-type epoxides postulated as intermediates in biosynthesis of the resolvin, protectin, and maresin lipid mediators formed from eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) fatty acids (4, 5). In fact, because *S*-lipoxygenases predominate in higher animal biology (45) and are implicated in the synthesis of the resolvin/protectin/maresin mediators, it is possible that novel transformations involving the LTA-type epoxides in higher animals follow the same relationships as in the 5*S*-LOX pathway to the leukotrienes. Isolation and identification of these intermediates are in progress to examine the structures experimentally.

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