Mutation of Tyrosine 383 in Leukotriene A4 Hydrolase
Allows Conversion of Leukotriene A4 into
5S,6S-Dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic Acid

IMPLICATIONS FOR THE EPOXIDE HYDROLASE MECHANISM*

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Leukotriene A4 hydrolase is a bifunctional zinc metalloenzyme that catalyzes the final step in the biosynthesis of the proinflammatory mediator leukotriene B4. In previous studies with site-directed mutagenesis on mouse leukotriene A4 hydrolase, we have identified Tyr-383 as a catalytic amino acid involved in the peptidase reaction. Further characterization of the mutants in position 383 revealed that [Y383H], [Y383F], and [Y383Q] leukotriene A4 hydrolases catalyzed hydrolysis of leukotriene A4 into a novel enzymatic metabolite. From analysis by high performance liquid chromatography, gas chromatography/mass spectrometry, gas chromatography/mass spectrometry of material generated in the presence of H218O or H216O, steric analysis of the hydroxyl groups, treatment with soybean lipase, and comparison with a synthetic standard, the novel metabolite was assigned the structure 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (5S,6S-DHETE). The kinetic parameters for the formation of this novel metabolite, termed 5S,6S-DHETE, were found to be similar. Also, both activities were susceptible to suicide inactivation and were equally sensitive to inhibition by bestatin. Moreover, from the stereochemical configuration of the vicinal diol, it could be inferred that 5S,6S-DHETE is formed via an Sn1 mechanism involving a carbocation intermediate, which in turn indicates that enzymatic hydrolysis of leukotriene A4 into leukotriene B4 follows the same mechanism. Inasmuch as soluble epoxide hydrolase utilizes leukotriene A4 as substrate to produce 5S,6R-DHETE, our results also suggest a functional relationship between leukotriene A4 hydrolase and xenobiotic epoxide hydrolases.

Leukotriene A4 (LTA4)3 hydrolase is a soluble enzyme that catalyzes the production of 5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (LTB4) from the transient allylic epoxide 5S-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid (LTA2) (1). In only nanomolar concentrations, LTB4 elicits chemotaxis and adherence of leukocytes, and in higher doses, it also triggers degranulation and generation of superoxide anions. Due to these biological properties, LTB4 is regarded as an important chemical mediator in a variety of inflammatory disorders. In the absence of epoxide hydrolase, LTA4 can undergo rapid spontaneous hydrolysis into the epimers at C-12 of 5S,12-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid (Δ8-trans-LTB4 and 12-epi-Δ8-trans-LTB4) and two diastereoisomers of 5,6-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (5,6-DHETE) (2).

Sequence comparisons of LTA4 hydrolase with other zinc metalloenzymes, e.g. aminopeptidase M and thermolysin, led to the identification of a zinc-binding motif (3, 4). Subsequently, the enzyme was shown to contain one catalytic zinc atom complexed to His-295, His-299, and Glu-318 (5–7). Furthermore, LTA4 hydrolase was found to exhibit an aminopeptidase activity toward synthetic substrates (6, 8). Although a physiological substrate has not yet been found, LTA4 hydrolase has been shown to efficiently hydrolyze several arginyl tri- and dipeptides (9).

From data obtained by site-directed mutagenesis and biochemical analysis of purified recombinant proteins, Glu-296, a residue conserved within the zinc-binding motif, was shown to be catalytic in the peptidase reaction, where it presumably acts as a general base (10). Such a reaction mechanism postulates a proton transfer to the nitrogen of the peptide bond (11, 12). Further sequence comparisons between LTA4 hydrolase and aminopeptidase M led to the identification of a conserved proton donor motif, and tyrosine 383 was suggested as the putative proton donor in LTA4 hydrolase (13). This finding prompted us to investigate the role of Tyr-383 for the catalytic activities of LTA4 hydrolase. Thus, the Tyr residue was exchanged for a Phe, His, or Gln residue in mouse LTA4 hydrolase by site-directed mutagenesis (14). The resulting proteins, [Y383F], [Y383H], and [Y383Q]LTA4 hydrolases, were expressed in Escherichia coli, purified to apparent homogeneity, and assayed for both activities. The results from this study showed that Tyr-383 is important for the peptidase activity, in which it may act as a proton donor. In contrast, Tyr-383 was not critical for the epoxide hydrolase activity, i.e., the conversion of LTA4 into LTB4. However, a more detailed characterization of these mutants revealed an additional enzyme activity for the substrate LTA4, as described in the present report. Thus, mutants in position 383, in particular [Y383Q]LTA4 hydrolase, converted LTA4 into 5S,6S-DHETE, the stereochemistry of which implies an Sn1 mechanism involving a carbocation intermediate in its formation. Since the mutants produced both
5S,6S-DHETE and LTB₄, this result indicates that the same mechanism applies to the enzymatic hydrolysis of LTA₄ into LTB₄. Inasmuch as soluble epoxide hydrolase accepts LTA₄ as substrate and converts it into an epimeric 5,6-DHETE, our data also suggest a functional link between LTA₄ hydrolase and xenobiotic soluble epoxide hydrolase.

EXPERIMENTAL PROCEDURES

Materials—LTA₄, ethyl ester (Merck-Frosst Labs, Pointe-Claire, Quebec, Canada) was saponified in tetrahydrofuran with 1 M LiOH (6%), by volume; 24 °C (4 °C). LTA₄, methyl ester was from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Betastatin, soybean lipoxygenase, and methyl (5Z)-eicosenoate were obtained from Sigma. Synthetic 5S,6S-DHETE was from Cayman Chemical Co., Inc. (Ann Arbor, MI), and HPLC solvents were purchased from Rathburn Chemicals (Walkerburn, United Kingdom).

Construction of Mutants—The mutants were produced by site-directed mutagenesis of the recombinant plasmid pULTA4, an E. coli expression vector containing the entire coding sequence for mouse LTA₄ hydrolase (15). The mutagenesis was carried out by a method based on polymerase chain reaction, and as described previously, the entire eDNA of all mutants were sequenced to confirm that no alterations other than the desired mutations had occurred (14).

Expression and Purification of Recombinant Enzymes—The mutant proteins were expressed in E. coli JM101 cells in M9 medium at 37 °C. After isopropyl-β-D-thiogalactopyranoside induction, the incubation was continued for 2–3 h, and the cells were collected by centrifugation and purified by fast protein liquid chromatography as described (16). The purity of the final enzyme preparations was assessed by SDS-polyacrylamide gel electrophoresis and Western blot analysis, which revealed essentially homogeneous proteins. The protein concentration was determined by the Bradford method (17) using bovine serum albumin as standard.

Incubations—Typically, aliquots of 10 μg of purified [Y383F], [Y383H], and [Y383Q]LTA₄ hydrolases in 100 μl of 50 mM Tris-Cl, pH 8.0, were incubated with the substrate LTA₄ (0.1 mM) at room temperature. After 15 s, the reaction was stopped by the addition of 2 volumes of methanol and a known amount of the internal standard prostaglandin B₁ (The Upjohn Co.). The samples were acidified to pH 3 by volume for 48 h at 10 °C/min until 240 °C. Injections were made in the split mode at an injector temperature of 250 °C. Helium at a flow rate of 38 cm/s was used as a carrier gas. Injections were made in the split mode at an injector temperature of 200 °C. The initial column temperature was 120 °C and was raised at 10 °C/min until 240 °C.

RESULTS

Incubation of Mutant Enzymes with LTA₄—Reverse-phase HPLC analysis of products formed when mutant enzymes, i.e., mouse [Y383F], [Y383H], and [Y383Q]LTA₄ hydrolases, were incubated with LTA₄, revealed five peaks (I–V) originating from the substrate (Fig. 1). Peaks I and II corresponded to the epimers at C-12 of 5S,12-dihydroxy-eicosanoate (50 μl) in anhydrous pyridine (50 μl) at 23 °C for 20 min. The reaction mixture was taken to dryness in vacuo, and the residue was suspended in hexane, centrifuged, and analyzed by GC/MS. The threo- and erythro-5,6-dihydroxyeicosanoates had retention times of 15.9 and 16.2 min, respectively, and exhibited similar mass spectra with prominent ions at m/z 471 (M⁺ – OCH₃), 299 (Me₅SiO⁺ – CH(CH₃)₃–CH₂–), and 203 (Me₃SiO⁺ – CH(CH₃)₂–COO–CH₃). Gas Chromatography/Mass Spectrometry—GC/MS was performed with a Hewlett-Packard Model 5970B mass selective detector connected to a Hewlett-Packard Model 5890 gas chromatograph equipped with a phenylmethysilicone capillary column (12-m length, 0.35-μm film thickness). Helium at a flow rate of 38 cm/s was used as a carrier gas. Injections were made in the split mode at an injector temperature of 200 °C. The initial column temperature was 120 °C and was raised at 10 °C/min until 240 °C.
For comparison, a sample of soluble epoxide hydrolase was incubated with LTA₄ to produce 5S,6R-DHETE (21), which did not, however, elute with compound V, but, as previously reported, with the more hydrophilic nonenzymatic isomer (Fig. 1).

**Structure of Compound V—**The material eluting under peak V was collected and subjected to UV spectrophotometry, which revealed a conjugated triene spectrum (in methanol) typical of leukotrienes, with a retention time of 15.9 min, and the mass spectrum showed prominent ions at m/z 471, 299, and 203. This retention time and mass spectrum were identical to those of the trimethylsilyl ether derivative of methyl 5,6-DHETE (20, 21), which most likely reflects differences in the experimental conditions and the instrumentation rather than structural differences of the compounds.

**Compound V was analyzed by GC/MS.** The mass spectrum of the methyl ester trimethylsilyl ether derivative had prominent ions at m/z 494 (M⁺), 479 (M - 15), 463 (M - 31), 404 (M - 90), 393 (M - 101; loss of -(CH₂)₅-COOCH₃), 291 (M - 203; loss of -(CH₂OSiMe₃)-(CH₂)₂-COOCH₃), 225, 203 (base peak; Me₅SiO⁺=CH-(CH₂)₃-COOCH₃), 171 (203 - 32), 147 (Me₅SiO⁺=SiMe₃), 129 (Me₅Si⁺=CH-CH=CH₂), and 113 (203 - 90) (Fig. 2), compatible with the presence of a tetraunsaturated C₂₀ fatty acid with hydroxyl groups at C-5 and C-6.

To determine the origin of the oxygen in the hydroxyl groups of compound V, incubations of [Y383(F/H/Q)]LTA₄ with 1.6 μg of compound V was incubated with 2.2 μg of soybean lipoxygenase in 500 μl of 0.1 M sodium borate buffer, pH 9, at room temperature. The time course for the reaction, measured as the increase in A₅₃₀, was followed on a UV spectrophotometer. The 5,6-DHETE triplet spectrum with a peak maximum at 275 nm in buffer (upper left inset) was completely shifted within 20 min to another triplet spectrum with a peak maximum at 303 nm, typical for a conjugated tetraene (lower right inset).

**Fig. 2. UV and mass spectra of compound V.** A, ultraviolet spectrum of compound V in methanol; B, mass spectrum of the methyl ester trimethylsilyl ether derivative of compound V.
synthetic standard of 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (Fig. 4). In addition, GC/MS analysis of the methyl ester trimethylsilyl ether derivative of the synthetic standard showed a practically identical chromatographic behavior and mass spectrum as compared with compound V (data not shown). Based on the data obtained from analysis by HPLC, UV spectrophotometry, GC/MS analysis of material generated in H216O and H218O, stereoechemical analysis of the hydroxyl groups, soybean lipoxigenase conversion, and comparison with a synthetic standard, compound V is assigned the structure 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid.

Catalytic Properties of Mutant Enzymes—The relative formation of 5S,6S-DHETE and LTB4 varied among the different mutants at position 383. Thus, at a LTA4 concentration of 26 μM, [Y383F], [Y383H], and [Y383Q]LTA4 hydrolases produced 66, 175, and 150% compound V, respectively (Table I). Also, the concentration of substrate varied between 7 and 80 μM. The wild-type enzyme exhibited a higher affinity for LTA4, and the wild-type enzyme (Table II). The Km values for the formation of 5S,6S-DHETE and LTB4 by [Y383Q]LTA4 hydrolase as well as the formation of LTB4, the mutant enzyme turns over LTA4 more efficiently (34 ± 103 s−1) than does the wild-type enzyme (kcat = 1.2). When LTA4 methyl ester (26 μM) was tested as substrate for [Y383Q]LTA4 hydrolase, no conversion into the esterified derivatives of LTB4 or 5S,6S-DHETE could be observed.

Suicide Inactivation and Sensitivity to Inhibition with Bestatin—[Y383Q]LTA4 hydrolase was tested for its susceptibility to suicide inactivation by LTA4. Aliquots of enzyme (10 μg in 100 μl of 50 mM Tris-Cl, pH 8.0) were incubated three times with 26 μM LTA4 for 30 min at room temperature (final concentration of 78 μM), followed by gel filtration and subsequent analysis of the remaining epoxide hydrolase activities, i.e. formation of LTB4 and 5S,6S-DHETE (Table III). In parallel, a sample of the wild-type enzyme was treated in the same way. As expected, the wild-type enzyme lost 50 and 58% of its peptidase and epoxide hydrolase activities, respectively, after exposure to LTA4. Similarly, [Y383Q]LTA4 hydrolase lost 50 and 58% of its ability to form LTB4 and 5S,6S-DHETE, respectively. Although we did not demonstrate covalent binding between LTA4 and [Y383Q]LTA4 hydrolase, the loss of enzyme activities indicates that the wild-type and mutant enzymes are equally susceptible to suicide inactivation by LTA4.

We also investigated the sensitivity of the two catalytic activities of [Y383Q]LTA4 hydrolase to inhibition by the competitive inhibitor bestatin. Thus, the concentrations of bestatin required for half-maximal inhibition (IC50) of the formation of 5S,6S-DHETE and LTB4 were 1.7 and 1.9 μM, respectively (Table IV). To circumvent potential differences in inhibitor potency related to differences in substrate affinity, the Ks values for inhibition of the two activities were calculated from Dixon plots. The Ks values of bestatin for the conversion of LTA4 into 5S,6S-DHETE and LTB4 by [Y383Q]LTA4 hydrolase were found to be 13 and 16 μM, respectively.

DISCUSSION

Recently, computer-assisted sequence comparisons and mutational analysis have been used to identify Tyr-383 as a potential proton donor in a general base mechanism for the peptidase activity of LTA4 hydrolase (13, 14). Since three mutants in position 383 converted LTA4 into LTB4, we concluded that Tyr-383 was not critical for the epoxide hydrolase activity of
LTA₄ hydrolase. However, a more detailed analysis of the catalytic consequences of the amino acid changes led to the unexpected finding that all mutant enzymes, and in particular [Y383Q]LTA₄ hydrolase, generated a second enzymatic product in addition to the expected LTB₄ (Fig. 1).

**Putative Mechanism for the Formation of 5S,6S-DHETE—**Several different approaches were used to solve the structure of the novel enzymatic metabolite, termed compound V. Its UV spectrum and retention time in reverse-phase HPLC indicated that it could be an isomer of 5,6-DHETE, an assumption that was verified by GC/MS analysis (Fig. 2). The mass spectrum of compound V, generated in a buffer containing H₂¹⁸O, revealed that the hydroxyl group at C-6 originated from water. Since the epoxide oxygen of LTA₄ is in the S-configuration at C-5, the stereochemistry of the vicinal diol must be either 5S,6S or 5S,6R. The latter alternative seemed unlikely since compound V did not cochromatograph with 5S,6R-DHETE (Fig. 1), the enzymatic product obtained when soluble epoxide hydrolase is incubated with LTA₄ (21). Final proof for the stereochemistry of the diol was obtained by GC/MS analysis of the trimethylsilyl ether derivative of a hydrogenated and methylated sample of compound V, which displayed a retention time and fragmentation pattern identical to those of an authentic standard of methyl three-5,6-dihydroxyeicosanoate, demonstrating that the hydroxyl groups at C-5 and C-6 must be in the S-configuration. Furthermore, evidence for the presence of two cis-double bonds at Δ¹₁ and Δ¹₂ was obtained by treatment of compound V with soybean lipoxigenase, which led to a 30-nm bathochromic shift of the UV spectrum into an absorbance profile typical of a conjugated tetraene (Fig. 3). Since soybean lipoxigenase requires a 1,4-cis-pentadiene structure for activity, it is reasonable to assume that 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid was converted into 5S,6S-dihydroxy-15S-hydroperoxy-7,9,13-trans-11-cis-eicosatetraenoic acid. Finally, compound V was found to have the same retention time in reverse-phase HPLC as a synthetic standard of 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (Fig. 4), and GC/MS analysis of the synthetic material gave virtually identical results as for compound V. Based on these analytical data and the known structure of the substrate LTA₄, compound V was assigned the tentative structure 5S,6S-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (Fig. 5).

**Table III**

| Enzyme | Residual epoxide hydrolase activity | Residual peptidase activity |
|--------|------------------------------------|-----------------------------|
|        | LTB₄ | 5S,6S-DHETE |                         |
| Wild-type | 58  | 50 | 50 |
| [Y383Q] | 50 | 58 |

**TABLE IV**

| IC₅₀ (μM) | LTB₄ formation |
|-----------|----------------|
| 1.7       | 1.9            |
| Kᵯ (μM)  | 13             |

**5S,6S-DHETE Is a Major Enzymatic Product Generated by Mutants of Tyr-383—**As mentioned above, all mutants in position 383 of mouse LTA₄ hydrolase converted LTA₄ into compound V, i.e., 5S,6S-DHETE. Considering the relative formation of 5S,6S-DHETE (versus LTB₄), the mutants had the following order of efficiency: [Y383F] > [Y383Q] > [Y383F] LTA₄ hydrolase (Table I). For [Y383Q]LTA₄ hydrolase, the mutant with the highest specific epoxide hydrolase activity, the relative formation of 5S,6S-DHETE versus LTB₄ was 150%, and thus, 5S,6S-DHETE was the dominating product (Fig. 5). This is noticeable since 5,12-dihydroxy acids are by far the most abundant metabolites obtained via nonenzymatic hydrolysis as well as for hydrolysis catalyzed by wild-type LTA₄ hydrolase, suggesting that the vicinal diols are thermodynamically unfavored products. Also, the relative formation of 5S,6S-DHETE by [Y383Q]LTA₄ hydrolase did not change when the concentration of substrate varied between 7 and 80 μM.

**[Y383Q]LTA₄ Hydrolase Turns over LTA₄ More Efficiently than Does the Wild-type Enzyme—**As previously reported, [Y383Q]LTA₄ hydrolase exhibits a higher Kᵯ for LTA₄ as compared with the wild-type enzyme, and at saturating concentrations of substrate, the specific epoxide hydrolase activity (considering only the formation of LTB₄) was estimated to be 60% of the control (14). However, if the formation of 5S,6S-DHETE is taken into account, the turnover of LTA₄ is in fact higher for [Y383Q]LTA₄ hydrolase, i.e., kₗᵃᵗ = 1.8 versus 1.2 s⁻¹ for the wild-type enzyme (Table II). When the kinetic data for the formation of LTB₄ and 5S,6S-DHETE by [Y383Q]LTA₄ hydrolase were plotted separately, no significant differences were observed, except for a somewhat higher Vₘ₉₅ value for the vicinal diol formation, as expected. The kinetic data listed in Table II indicate a difference in Kᵯ for LTA₄. However, due to the instability of LTAs, determinations of the Michaelis constant become very uncertain and difficult to evaluate. Thus, it is questionable whether a 2-fold increase in Kᵯ is really significant.

**Putative Mechanism for the Formation of 5S,6S-DHETE—**All mutants of Tyr-383 catalyzed the stereospecific addition of water at two positions, i.e., C-6 and C-12, in the substrate LTA₄. This unusual catalytic behavior can be explained in several ways. The mutant enzymes may use two separate functional elements toward either of the two positions of the substrate, which in turn would require that the respective mutation “activate” an additional structure within the active site to perform a nucleophilic attack on the substrate (Fig. 6). Alternatively, one functional element can operate on both positions. The latter and perhaps more plausible option would be possible if the substrate could enter the active site in two opposite head-
methyl ester was not converted into the methyl ester of 5S,6S-DHETE lends further support to this interpretation. An additional possibility would be a single binding mode for the substrate in an active site with one functional element controlling the stereospecific hydrolysis leading to both products. Although LTA₄ would bind in one orientation to the substrate-binding pocket, it may still have a conformation and position in space that make C-12 and C-6 available for attack by a single nucleophile (Fig. 6).

Evidence for the Presence of a Carbocation Intermediate in the Enzymatic Hydrolysis of LTA₄—From experiments with H₂¹⁸O, it was inferred that the nucleophilic attack of water during the formation of 5S,6S-DHETE was directed toward C-6 according to an S₀₁ reaction. Considering the S-configuration of the hydroxyl group at C-6 in S₀₂ reaction, with concomitant chiral inversion, would not be possible since the epoxide oxygen of the substrate is already in the 6S-configuration. Thus, enzymatic hydrolysis of LTA₄ into 5S,6S-DHETE must occur via an S₀₁ reaction involving a carbocation intermediate. This intermediate would be planar at C-6, allowing an enzyme-directed nucleophilic attack from either side of the molecule. Since [Y₃₈₃F], [Y₃₈₃H], and [Y₃₈₃Q]LTA₄ hydrolases could produce not only 5S,6S-DHETE, but also LTB₄, it seems very likely that hydrolysis of LTA₄ into LTB₄ proceeds according to the same mechanism. This conclusion was further corroborated by the fact that [Y₃₈₃Q]LTA₄ hydrolase makes both products with indistinguishable reaction kinetics. Nevertheless, we cannot rule out the possibility that LTB₄ could be formed via an S₀₂ or rather S₀₂⁻ reaction, an interpretation that seems unlikely, however, since it would imply that the mutants can operate simultaneously at C-6 and C-12 of LTA₄ via two distinct enzyme mechanisms. Hence, assuming that the mutations do not interfere with the fundamental enzyme mechanism for the epoxide hydrolysis, the formation of a vicinal diol with the stereochemical configuration 5S,6S by the mutant enzymes represents the first experimental evidence that the formation of LTB₄ by wild-type LTA₄ hydrolase follows an S₀₁ mechanism involving a carbocation intermediate. In addition, this reaction mechanism would be in agreement with the mechanism for nonenzymatic hydrolysis of LTA₄ (2) and conforms to the general rule that enzymes reduce the activation energy for chemical reactions that also occur spontaneously (23).

Possible Function of Tyr-383—We have recently identified a 21-residue peptide segment, denoted peptide K21, to which LTA₄ binds during suicide inactivation (24), and amino acid sequence analysis of a covalently modified form of peptide K21, isolated from human LTA₄ hydrolase inactivated by LTA₄ ethyl ester, indicated that Tyr-378 is a primary site for covalent binding of lipid to the protein. This conclusion was further corroborated by mutational analysis, which revealed that exchange of Tyr-378 for a Phe or Gln rendered the enzyme virtually resistant to mechanism-based inactivation (25). Furthermore, Tyr-378 appeared to be involved in the formation of the correct double bond geometry in LTB₄ since both of these mutants, human [Y₃₈₃F] and [Y₃₈₃Q]LTA₄ hydrolases, catalyzed the formation of a second enzymatic product from LTA₄, viz. Δ⁸-trans-Δ⁸-cis-LTB₄ (26). Notably, Tyr-383 is also located within peptide K21, separated from Tyr-378 by only four residues. Hence, each of the two tyrosines is involved in one of the two main catalytic features of LTA₄ hydrolase, i.e. stereospecific hydrolysis at C-12 and creation of a cis,trans,trans-epoxide oxygen of the substrate is already in the 6S-configuration. Thus, enzymatic hydrolysis of LTA₄ into 5S,6S-DHETE must occur via an S₀₁ reaction involving a carbocation intermediate. This intermediate would be planar at C-6, allowing an enzyme-directed nucleophilic attack from either side of the molecule. Since [Y₃₈₃F], [Y₃₈₃H], and [Y₃₈₃Q]LTA₄ hydrolases could produce not only 5S,6S-DHETE, but also LTB₄, it seems very likely that hydrolysis of LTA₄ into LTB₄ proceeds according to the same mechanism. This conclusion was further corroborated by the fact that [Y₃₈₃Q]LTA₄ hydrolase makes both products with indistinguishable reaction kinetics. Nevertheless, we cannot rule out the possibility that LTB₄ could be formed via an S₀₂ or rather S₀₂⁻ reaction, an interpretation that seems unlikely, however, since it would imply that the mutants can operate simultaneously at C-6 and C-12 of LTA₄ via two distinct enzyme mechanisms. Hence, assuming that the mutations do not interfere with the fundamental enzyme mechanism for the epoxide hydrolysis, the formation of a vicinal diol with the stereochemical configuration 5S,6S by the mutant enzymes represents the first experimental evidence that the formation of LTB₄ by wild-type LTA₄ hydrolase follows an S₀₁ mechanism involving a carbocation intermediate. In addition, this reaction mechanism would be in agreement with the mechanism for nonenzymatic hydrolysis of LTA₄ (2) and conforms to the general rule that enzymes reduce the activation energy for chemical reactions that also occur spontaneously (23).

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Notably, apart from the three zinc-binding ligands, Tyr-383 is the only amino acid residue identified thus far that seems to be involved in both the peptidase and epoxide hydrolase activities of LTA₄ hydrolase, thus bridging the two corresponding active sites. It should also be noted that the results concerning Tyr-378 were obtained with the human LTA₄ hydrolase, whereas the data in the present report were obtained with the mouse enzyme. However, since the human and mouse LTA₄ hydrolases are 93% identical at the amino acid level, it is reasonable to assume that results obtained by mutational analysis in one of the species pertain to the other.

Functional Relationship to Soluble Xenobiotic Epoxide Hydrolase—Apart from LTA₄ hydrolase, there are at least two other mammalian epoxide hydrolases that have been extensively characterized, i.e., soluble and microsomal epoxide hydrolases (27, 28). Both of these enzymes are believed to be involved in the detoxification of potentially harmful xenobiotic epoxides, although several endogenous physiological substrates have been described, at least for soluble epoxide hydrolase (21, 29–32). Recent work including computer-assisted sequence comparisons, x-ray crystallographic analysis of structurally related enzymes, and biochemical and mutational analyses has identified soluble and microsomal epoxide hydrolases as members of the α/β-fold family of hydrolases (33–39). Concerning LTA₄ hydrolase, there is not enough structural or biochemical data available to conclusively determine whether or not it belongs to the same class of enzymes. However, LTA₄ has been shown to be an excellent substrate for soluble epoxide hydrolase (but not for microsomal epoxide hydrolase), which converts the allylic epoxide into 5S,6S-DHETE, i.e. an epimer at C-6 of the vicinal diol produced by [Y383Q]LTA₄ hydrolase (21). Hence, the subtle structural changes at the active site of [Y383Q]LTA₄ hydrolase shift the positional specificity of the stereospecific hydrolase such that the mutant enzyme begins to mimic the action of soluble epoxide hydrolase. One may speculate that this functional resemblance, caused by a single amino acid change, is a sign of structural similarity between the active sites of soluble epoxide hydrolase and LTA₄ hydrolase.

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