Leukotriene A₄ Hydrolase, Mutation of Tyrosine 378 Allows Conversion of Leukotriene A₄ into an Isomer of Leukotriene B₄

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Leukotriene A₄ hydrolase catalyzes the final step in the biosynthesis of the proinflammatory compound leukotriene B₄, a reaction which is accompanied by suicide inactivation of the enzyme by leukotriene A₄. We have recently reported that Tyr-378 is a major structural determinant for suicide inactivation and that mutation of Tyr-378 into Phe or Gln protects leukotriene A₄ hydrolase from this catalytic restriction (Mueller, M. J., Blomster, M., Opperman, U. C. T., Jörnvall, H., Samuelsson, B., and Haeggström, J. Z. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5931–5935). In the present study, we show that both [Y378F]- and [Y378Q] leukotriene A₄ hydrolase converts leukotriene A₄ not only into leukotriene B₄ but also into a second, previously unknown, product of the enzyme. From biophysical analyses and comparison with a synthetic standard, the structure of this product was determined to 5S,12R-dihydroxy-6,14-cis-eicosatetraenoic acid, i.e. Δ⁴-trans-Δ⁸ cis-leukotriene B₄. The relative formation of Δ⁴-trans-Δ⁸ cis-leukotriene B₄ versus leukotriene B₄ by [Y378F]- and [Y378Q] leukotriene A₄ hydrolase, was 18% and 32%, respectively. For [Y378F] leukotriene A₄ hydrolase, the turnover of leukotriene A₄ into leukotriene B₄ or Δ⁴-trans-Δ⁸ cis-leukotriene B₄ was calculated to 2.5 s⁻¹ which is almost three times the kₘ value of the wild type enzyme. Taken together, these findings indicate that Tyr-378 is located at the active site where it assists the product leukotriene A₄ hydrolase. From biophysical analyses and comparison with a synthetic standard, the structure of this product was determined to 5S,12R-dihydroxy-6,14-cis-eicosatetraenoic acid, i.e. Δ⁴-trans-Δ⁸ cis-leukotriene B₄. The relative formation of Δ⁴-trans-Δ⁸ cis-leukotriene B₄ versus leukotriene B₄ by [Y378F]- and [Y378Q] leukotriene A₄ hydrolase, was 18% and 32%, respectively. For [Y378F] leukotriene A₄ hydrolase, the turnover of leukotriene A₄ into leukotriene B₄ or Δ⁴-trans-Δ⁸ cis-leukotriene B₄ was calculated to 2.5 s⁻¹ which is almost three times the kₘ value of the wild type enzyme. Taken together, these findings indicate that Tyr-378 is located at the active site where it assists the formation of the correct double-bond geometry in the product leukotriene B₄.

Leukotriene (LT) A₄ hydrolase (EC 3.3.2.6) is a bifunctional zinc metalloenzyme which converts LTA₄ into the proinflammatory substance LTB₄, a reaction referred to as the epoxide hydrolase activity (1). In addition, LTA₄ hydrolase possesses an anion-dependent aminopeptidase activity (2–4), the physiological significance of which is still unknown.

During the epoxide hydrolysis, LTA₄ hydrolase is covalently modified and inactivated by its endogenous lipid substrate LTA₄ via an apparently mechanism-based process, also referred to as suicide inactivation (5–8). Notably, suicide inactivation is accompanied by loss of both the epoxide hydrolase and the aminopeptidase activity, in agreement with the notion that the corresponding active site(s) share important structural and/or functional elements.

We have recently identified a peptide segment encompassing residues 365–385 in LTA₄ hydrolase to which LTA₄ binds during suicide inactivation (9). A tyrosine residue in position 378 within this peptide appeared to be a primary site for the covalent binding of the 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 (10). When the mutated enzymes [Y378F]- and [Y378Q] LTA₄ hydrolase were studied in greater detail, it became apparent that they were able to catalyze hydrolysis of LTA₄ not only into the expected product LTB₄, but also into a novel, previously unknown, enzyme metabolite, as described in the present work. This finding indicates that Tyr-378 is an active site residue, and, in addition, the structure of the novel product gives a clue as to the function of Tyr-378 in the enzymatic conversion of LTA₄ into LTB₄.

EXPERIMENTAL PROCEDURES

Materials—LTA₄ ethyl ester (Merck-Frosst Laboratories, Pointe Claire, QC, Canada) was saponified in tetrahydrofuran with 1 M LiOH (6% w/v) for 48 h at 4 °C. Alanine-4-nitrosoamide was from Sigma and 12R-hydroxy-5,8,14-cis-trans-eicosatetraenoic acid (12R-HETE) from Cascade.

Preparation of Mutated Enzymes—The mutants [Y378F]-, [Y378Q]-, [S379A]-, and [S380A] LTA₄ hydrolase were constructed by polymerase chain reaction mutagenesis, expressed as (His)_₄-linked fusion proteins in Escherichia coli (JM101) cells, and purified by affinity chromatography on a nickel-nitrilotriacetic acid resin followed by chromatography on hydroxyapatite, as described previously (10).

Enzyme Assays—The epoxide hydrolase activity was determined by incubation of enzyme with LTA₄ followed by reverse-phase high performance liquid chromatography (HPLC) analysis of products, as described (11). Quantitations were based on peak area measurements, normalized with respect to the internal standard prostaglandin B₂ (PGB₂), using Baseline 810 computer software. The aminopeptidase activity was assayed spectrophotometrically in 50 mm Tris-Cl, pH 7.5, containing 100 μm NaCl and 38 μg/ml bovine serum albumin, using 1 μm alanine-4-nitrosoamide as the substrate (11).

Gas Chromatography-Mass Spectrometry—Gas chromatography linked to 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 methyl silicone capillary column (length 12 m, film thickness 0.33 μm). Helium at a flow rate of 36 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 GCMS, samples were converted to the methyl ester, trimethylsilyl ether derivatives, by treatment with diazomethane in diethyl ether followed by a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine.

Enzymatic Synthesis of Δ⁶-trans-Δ⁸-cis-LTB₄—SS,12R-Dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (Δ⁶-trans-Δ⁸-cis-LTB₄) was synthesized from 12R-hydroxy-5,8,14-trans-eicosatetraenoic acid (12R-HETE) using recombinant 5-lipoxygenase. A solution of 12R-HETE in ethanol was blown to dryness under a stream of argon and dissolved in 55 μl of 50 mM Tris-Cl, pH 7.5, containing EDTA and β-mercaptoethanol (buffer A). Stabilized 5-lipoxygenase (12) in 5 μl of buffer A was added, followed by 40 μl of the substrate mixture containing CaCl₂, ATP, and phosphatidylcholine in 50 mM Tris-Cl, pH 7.5, to start the reaction and the sample was incubated for 1 h at room temperature. The reaction mixture (total volume 100 μl) contained 2 μg of 12R-HETE, 10 μM 13-hydroperoxy-9,11-octadecadienoic acid (activator), 2.5 mM ATP, 1.92 mM CaCl₂, 1.2 mM EDTA, 0.025 mg/ml phosphatidylcholine, 15 mM β-mercaptoethanol, 75 mM Tris-Cl, pH 7.5, and 2.4 μg of 5-lipoxygenase. The generated 55-hydroperoxy-12R-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid was reduced by exposure to a small amount of NaBH₄ for 2 min. The reaction mixture was then subjected to solid-phase extraction (Chromabond C₁₈, 100 mg, Macherey-Nagel) and Δ⁶-trans-Δ⁸-cis-LTB₄ was purified by reverse-phase HPLC on a column (Novapak C₁₈, 4-μm Radial-Pak cartridge, 5 × 100 mm, Waters) eluted with a mixture of acetonitrile/methanol/water/acetic acid (30:35:35:0.1, v/v) at a flow rate of 1.2 mL/min. Quantitations were performed by integration of the peak area assuming an extinction coefficient of 40,000 M⁻¹ cm⁻¹ for Δ⁶-trans-Δ⁸-cis-LTB₄, and the recovery was calculated to 0.6 μg (30% overall yield).

RESULTS AND DISCUSSION

Previous studies have documented that LTA₄ hydrolase undergoes suicide inactivation during catalysis in a manner indicating a mechanism-based process (7, 8). We have recently identified a 21-residue peptide segment (denoted peptide K21) within LTA₄ hydrolase to which LTA₄ binds during suicide inactivation (9). Furthermore, amino acid sequence analysis of a covalently modified form of K21, isolated from LTA₄ hydrolase inactivated by LTA₄ ethyl ester, indicated that Tyr-378 is a primary site for covalent binding of lipid to the protein. To detail the role of Tyr-378 in mechanism-based inactivation of LTA₄ hydrolase, this residue, together with two adjacent serine residues, were subjected to mutational analysis. Interestingly, mutants of Tyr-378, i.e. [Y378F]- and [Y378Q]-LTA₄ hydrolase, but not mutants of Ser-379 or Ser-380, i.e. [S379A]- and [S380A]-LTA₄ hydrolase, were found to be resistant to inactivation by LTA₄ (10). A more detailed examination of the catalytic properties of these mutants revealed that both [Y378F]- and [Y378Q]-LTA₄ hydrolase were able to generate a second metabolite of LTA₄.

Novel Catalytic Activity of Mutants of Tyrosine 378—Reverse-phase HPLC analysis of products formed when [Y378F]- and [Y378Q]-LTA₄ hydrolase were incubated with LTA₄ revealed the presence of an unknown peak, termed compound IV, which eluted shortly after LTB₄ (Fig. 1). The UV spectrum of compound IV in MeOH of compound IV. B, mass spectrum of compound IV. C, UV-induced isomerization of compound IV and 5S,12S-DiHETE into the all-trans isomers of LTB₄. Alloquin of compound IV (1 nmol in 100 μl of Tris-Cl, pH 8) or 5S,12S-DiHETE were irradiated with UV light at 310 nm for 90 s, followed by reverse-phase HPLC analysis. From top to bottom, the figure depicts the chromatograms obtained with untreated compound IV (a), irradiated compound IV (b), untreated 5S,12S-DiHETE (c), irradiated 5S,12S-DiHETE (d), and a mixture of the internal standard PGB₁, Δ⁶-trans-LTB₄, Δ⁸-trans-12-epi-LTB₄, and compound IV (e).
The specific epoxide hydrolase activity, defined as the hydrolysis of LTA₄ into LTB₄, alone (wild type enzyme) or a mixture of LTB₄ and Δ⁶-trans-Δ⁸-cis-LTB₄ (3), was determined from short-time (15-s) incubations of the respective enzyme (2 μg) dissolved in 200 μl of 50 mM Tris-Cl, pH 8, with 13 μM LTA₄ at room temperature. The products LTB₄ and Δ⁶-trans-Δ⁸-cis-LTB₄ were quantitated with reverse-phase HPLC. The specific peptidase activity was determined from incubations of 2 μg of the respective enzyme with 1 mM alanine-4-nitroanilide in 50 mM Tris-Cl, pH 7.5, containing 100 mM NaCl and 38 μg/ml bovine serum albumin. Formation of 4-nitroaniline was determined by spectrophotometric analysis at 405 nm.

Structure of Compound IV—Compound IV was analyzed by GC/MS. The mass spectrum had characteristic ions at m/z 402 (M - 90), 354, 213 (Me₂SiO=CH-CH₂-CH=CH-CH₂), 203 (Me₂SiO=CH-CH₂-CH=CH₂), 171 (203 - 32; loss of CH₂OH), and 129 (Me₂SiO=CH-CH=CH₂), compatible with a 5,12-dihydroxyeicosatetraenoic acid (Fig. 2). In GC, compound IV was unstable and eluted as a tailing peak particularly in recordings of the selected ion current of m/z 213, as previously reported for 5S,12S-DiHETE (13). This GC behavior and the appearance of ion m/z 213 at high relative intensity, is due to a thermal rearrangement which is typical for compounds with a trans-cis-trans conjugated triene structure (13).

To establish the stereochemistry of the hydroxyl groups of compound IV, conjugated cis-double bonds were isomerized, by a short time (<90 s) exposure to UV light at 310 nm, to produce one of the all-trans isomers of LTB₄. As shown in Fig. 2, the unknown compound IV was isomerized predominantly into 5S,12R-dihydroxy-6,8,10-trans-14-eicosaetraenoic acid (Δ⁶-trans-LTB₄), which was also obtained after isomerization of LTB₄ (data not shown). As a control, the Δ⁸-cis double bond of 5S,12S-dihydroxy-6,10-trans-8,14-eicosatetraenoic acid (5S,12S-DiHETE) was isomerized which generated the expected product 5S,12S-dihydroxy-6,8,10-trans-14-eicosatetraenoic acid (Δ⁶-trans-12-epi-LTB₄). Thus, the isomerization experiment of compound IV showed that the stereochemistry of the hydroxyl groups must be the same as in LTB₄, i.e. 5S,12R, and that compound IV has at least one cis double bond probably at Δ⁸ or Δ⁶, since it is different from LTB₄ and its all-trans isomers (compounds I–III, Fig. 1). In principal, a number of possible configurations have to be considered for the double-bond geometry of the conjugated triene of compound IV. However, considering the GC/MS data which indicated the presence of a trans-cis-trans conjugated triene (see above), a single cis double bond at Δ⁸ seems to be the most likely alternative. The UV spectrum of compound IV (λₘₐₓ at 259, 268, and 279 nm) lendereadier support for this conclusion. Thus, previous studies have shown that the UV-spectra of LTB₄ and Δ⁶-trans-Δ⁸-cis-LTB₄ are essentially identical with λₘₐₓ = 270 nm whereas isomers carrying a Δ⁶-trans-Δ⁸-cis-Δ⁸-trans conjugated triene system exhibit spectra with λₘₐₓ = 268 nm (14–16).

To make possible a direct comparison, a standard of Δ⁶-trans-Δ⁸-cis-LTB₄ was synthesized from 12R-HETE. 5-Lipoxygenase is known to oxygenate 12S-HETE stereospecifically to form 5S,12R-DiHETE. In analogy, we used 12R-HETE as a substrate to produce Δ⁶-trans-Δ⁸-cis-LTB₄ which showed the same UV spectrum, the same chromatographic behavior in HPLC and GC and the same MS fragmentation pattern as compound IV (cf. Figs. 1 and 2). Based on these data, compound IV was assigned the tentative structure 5S,12R-dihydroxy-6,10-trans-8,14-eicosatetraenoic acid (Fig. 3).

A compound with this structure was recently identified in organ homogenates of the African claw toad Xenopus laevis, incubated with LTA₄ (17). In view of the results of the present investigation, it is tempting to speculate that the compound perhaps originated from the action of an isoform of LTA₄ hydrolase present in Xenopus tissues.

### Table I

| Epoxide hydrolase activity | Peptidase activity |
|---------------------------|--------------------|
| nmol/mg/min               | %                  |
| Wild type                 | 100 ± 0.9          |
| [Y378F]                   | 203 ± 4            |
| [Y378Q]                   | 45 ± 2             |

| Wild type                 | 188 ± 8            |
| [Y378F]                   | 81 ± 4             |
| [Y378Q]                   | 3 ± 0.3            |

| Peptidase activity mean ± S.D. (n = 3) | %                  |
|---------------------------------------|--------------------|
| Wild type                             | 100 ± 0.9          |
| [Y378F]                               | 43 ± 2             |
| [Y378Q]                               | 1.6 ± 0.2          |

### Table II

#### Apparent kinetic constants for hydrolysis of LTA₄ and alanine-4-nitroanilide by wild type and [Y378F]/[Y378Q] LTA₄ hydrolase

| Epoxide hydrolase activity | Peptidase activity |
|----------------------------|--------------------|
|                          | Wild type          |
|                          | [Y378F]            |
|                          | [Y378Q]            |
| Kₘ (μM)                  | 5.8                |
| Vₘₐₓ (nmol mg⁻¹ min⁻¹)   | 735                |
| kₜₚ (s⁻¹)                | 0.85              |
| kₜₚ/Kₘ (s⁻¹ μM⁻¹)        | 147 × 10⁻³         |

|                          | 23.1               |
|                          | 2126              |
|                          | 2.46              |
|                          | 107 × 10⁻¹         |
|                          | 447               |

|                          | 3300               |
|                          | 183.5              |
|                          | 0.21              |
|                          | 58.3              |

| a | The values of Kₘ refer to the specificity constant for the substrate alanine-4-nitroanilide. |
| b | For determination of Vₘₐₓ for [Y378F]/[Y378Q] LTA₄ hydrolase, the sum of LTB₄ and Δ⁶-trans-Δ⁸-cis-LTB₄ was used in calculations of product formation. |
Possible Role of Tyr-378 in the Hydrolysis of LTA₄ into LTB₄—Leukotriene A₄ is a highly unstable allylic epoxide which is spontaneously hydrolyzed in water with a $t_{1/2} \approx 10$ s at neutral pH (18). Nonenzymatic hydrolysis of LTA₄ is thought to be initiated via an acid-induced opening of the epoxide moiety (19) and a carbonium ion, with a positive charge delocalized over the triene system, would be formed as an intermediate in the reaction. This intermediate will result in a planar $sp^2$-hybridized configuration at C12, which in turn allows a nucleophilic attack from both sides of the carbon. Accordingly, the two epimers at C12 of 5S,12-dihydroxy-6,8,10-trans-14-cis-eicosa- tetraenoic acid, also referred to as $\Delta^6$-trans-LTB₄ and $\Delta^8$-trans-12-epi-LTB₄ will be formed and are in fact the predominant nonenzymatic hydrolysis products of LTA₄. The structure of LTB₄, formed by enzymatic hydrolysis, differs from the structure of either of the two nonenzymatically formed 5,12-dihydroxy acids in two ways, viz. the double-bond geometry and the configuration of the hydroxyl group at C12. Apparently, during enzymatic hydrolysis of LTA₄ into LTB₄, LTA₄ hydrolase ensures a stereoselective introduction of H₂O at C12 as well as the formation of the thermodynamically less favored $\Delta^6$-cis-$\Delta^8$-trans-$\Delta^{10}$-trans configuration of the conjugated triene. Interestingly, the mutants at position 378 differ from wild type enzyme regarding one of these two essential functions of the enzyme, i.e. the positioning of the cis double bond in the product. Hence, Tyr-378 appears to be involved in this aspect of catalysis, perhaps by assisting in the proper alignment of LTA₄ in the substrate-binding pocket or by promoting a favorable conformation of a putative carbonium ion intermediate. Moreover, the present data, together with our previous findings regarding the role of Tyr-378 in suicide inactivation, strongly indicates that Tyr-378 is an active-site residue.

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