Enzymic Conversion of 11,12-Leukotriene A₄ to 11,12-Dihydroxy-5,14-cis-7,9-trans-eicosatetraenoic Acid

PURIFICATION OF AN EPOXIDE HYDROLASE FROM THE GUINEA PIG LIVER CYTOSOL

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Mammalian tissues possess at least three different types of arachidonic acid lipooxygenases (1, 2). 5-Lipooxygenase (3-6) synthesizes leukotriene A₄ (LTA₄)¹ which is further converted to LTB₄ (7-9) and LTC₄ (10-12). 15-Lipooxygenase products play an intermediary role in the formation of lipoxin (13). The biological significance of 12-lipooxygenase, abundant in various tissues (14-18), remains obscure. Although several investigators detected 11,12-di-hydroxyeicosatetraenoic acid (11,12-diHETE), as a possible metabolite of 12-lipooxygenase (1, 19), the absolute configuration of the compound and the direct evidence of its enzymic synthesis have not so far been demonstrated.

We report herein that 11,12-LTA₄ is nonenzymically decomposed to at least seven compounds, and that one stereoisomer of 11,12-diHETE is enzymically synthesized by epoxide hydrolase (EC 3.3.2.3) present in the various tissues of the guinea pig liver.

RESULTS

Enzymic Synthesis of C-3 from 11,12-LTA₄—When 11,12-LTA₄ was incubated in the standard reaction mixture without enzyme, seven compounds (Fig. 1A, peaks C-1, -2, -3 (two components), -4, -5, and -6) were observed and all showed characteristic UV spectra of conjugated triene with λₘₐₓ at 268 nm. Under present assay conditions, C-5 and C-6 were major products. When the guinea pig liver cytosol was added to the above reaction mixture, a prominent increase in C-3 was observed with a concomitant decrease in both C-5 and C-6 (Fig. 1B). As shown in Fig. 2, the 11,12-diHETE (C-3, see below) formation was increased dose-dependently on the enzyme amount, whereas the formations of both C-5 and C-6 were decreased. Thus, C-3 was enzymically synthesized from 11,12-LTA₄, and C-5 and C-6 were nonenzymic products.

Structural Identification of Products Derived from 11,12-LTA₄—The methyl esters of C-1 to -4 were obtained by the acid hydrolysis of 11,12-LTA₄, methyl ester (34), but those of C-5 and C-6 were not observed. C-1 and C-2 have already been identified (34) as the diastereomers of (5,12S)-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid ((5,12S)-dihETE).

C-3 in Fig. 1 gave two peaks by gas chromatography

EXPERIMENTAL PROCEDURES²

1 Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 3 and 4, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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3 To whom all correspondence and reprint requests should be addressed.

4 The abbreviations used are: LTA₄, (5S,6S)-epoxy-7,9-trans-11,14-cis-eicosatetraenoic acid; LT, leukotriene; 11,12-LTA₄, (11S,12S)-epoxy-5,14-cis-7,9-trans-eicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; diHETE, dihydroxyeicosatetraenoic acid; 12-HETE-4-lactone, 12-hydroxy-6,8,10,14-eicosatetraenoic acid; PGB₉, prostaglandin B₉; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

5799
The natural occurrence of 11,12-diHETE has been reported (1, 19), but the pathway of its formation and the absolute configuration of this compound have not so far been reported. The present study clearly showed that 11,12-diHETE was enzymatically synthesized from 11,12-LTA₄ by the action of the cytosolic epoxide hydrolase. The absolute configuration of the enzymic product was assigned as followed. The gas chromatography-mass spectrometry revealed that it was 11,12-dihydroxy-5,14-cis-7,9- eicosatetraenoic acid (see “Discussion”).

11,12-LTA₄ was incubated in the standard reaction mixture without enzyme for 1 min and the pH of the mixture was brought to 12 with 1 N KOH. The peaks of C-5 and C-6 disappeared with simultaneous increases of C-1 and C-2. C-5 was purified by reversed-phase HPLC, and the solvent was removed by lyophilization. It was subjected to fast atom bombardment-mass spectrometry. Molecular ions were observed at 319 and 320, with glycerol and [1H₆]glycerol as matrix, respectively. These data indicated that C-5 has one hydroxy moiety with an atomic composition of C₁₅H₂₉O₃. By IR spectrometry, an absorption at 1,730 cm⁻¹ was observed. C-6 gave essentially identical results as described for C-5. All these results strongly suggest that both C-5 and C-6 have a δ-lactone structure of 5,12-dihETE (C-1 and C-2). The mechanism of the δ-lactone formation will be discussed later.

**Catalytic Activities** — The purified enzyme catalyzed the formation of 11,12-diHETE. The time course was linear for at least 5 min. An optimum pH for the reaction was around 7.3. N-Ethylmaleimide and p-hydroxymercuribenzoic acid inhibited the enzyme activity with IC₅₀ values of 5 and 0.06 mM, respectively. It acted on LTA₄ and 14,15-LTA₄ in addition to 11,12-LTA₄. Among them, 11,12-LTA₄ was the best substrate in terms of both Kₘ and Vₘₐₓ values (Table IV).

The purified enzyme, also acted on the stable xenobiotic epoxides (2,3-disubstituted oxirans) to yield corresponding trans-opening hydrolysis products (1,2-glycols). The purified enzyme did not synthesize any detectable amount of the cis-opening hydrolysis products. The Vₘₐₓ value for trans-β-methylstyrene oxide was 1.2 times higher than that for 11,12-LTA₄ (Table IV). However, the Kₘ value (833 μM) for trans-β-methylstyrene oxide was more than 1 order of magnitude higher than that for 11,12-LTA₄ (18 μM) (Table IV). The 2,3-diphenyl-substituted oxirans, cis- and trans-stilbene oxides, were poorer substrates than 11,12-LTA₄ (Table V). The trans-isomer was hydrolyzed 3.2 times faster than the cis-isomer by the purified enzyme. Chalcone oxide (20 μM) showed a potent inhibitory effect on the hydrolysis of 11,12-LTA₄ and the xenobiotic 2,3-disubstituted oxirans, while it showed only a little effect on the hydrolysis of styrene oxide by the liver cytosol.

**DISCUSSION**

Fig. 2. Dependence of C-3 formation on the enzyme amount. The cytosol was dialyzed against Buffer I. A UV absorbance at 270 nm was monitored (~). A, 11,12-LTA₄ (1.5 nmol) was incubated in 100 mM Tris-HCl buffer, pH 7.3, containing 10 mg/ml bovine serum albumin at 37 °C for 1 min, and solution A was added to terminate the reaction according to “Experimental Procedures.” Prostaglandin B₉ was added as an internal standard (IS). B, 11,12-LTA₄ was incubated with guinea pig liver cytosol (0.6 mg of protein) at 37 °C for 1 min. Peaks C-1 to C-6 and *showed UV absorption of the conjugated triene. The peak * had the same retention time as C-1 methyl ester, possibly derived from unreacted substrate (11,12-LTA₄ methyl ester). C-2 methyl ester coeluted with C-5.

The geometry of double bonds was assigned as 5,14-cis-7,9-trans by proton NMR (Table I). Although the stereochemistry of C₁₁ and C₁₂ was not fully identified, C-3 is assigned to be (11R,12S)-dihydroxy-5,14-cis-7,9-trans eicosatetraenoic acid (see “Discussion”).
purification of the enzyme to homogeneity by the use of a different from other cytosolic epoxide hydrolases, the guinea pig enzyme was extremely hydrophobic. We succeeded in the guinea pig liver cytosol.

Cytosolic epoxide hydrolases have been purified from livers of rabbit (36), mouse (37, 44) and human (43). Although a similar enzyme activity was demonstrated in the guinea pig liver (23, 44), the enzyme was not heretofore purified and a similar enzyme activity was demonstrated in the guinea pig liver (23, 44). The enzyme was not heretofore purified and a similar enzyme activity was demonstrated in the guinea pig liver. In fact, the antibody against mouse liver cytosolic epoxide hydrolase did not react immunologically with the cytosol of guinea pig (46). Though all these enzymes are collectively termed "xenobiotic epoxide hydrolase," the enzyme from other species heterogeneity or to the presence of different enzyme.

we concluded that the structure of the enzymically synthesized product is (11R,12S)-dihydroxy-5,14-cis-7,9-trans-eicosatetraenoic acid. It was demonstrated in the present study that 11,12-diHETE, a previously reported compound (1, 19), was synthesized from 11,12-LTA4 by the epoxide hydrolase in the guinea pig liver cytosol.

The enzyme activities were measured according to procedures described under "Experimental Procedures" with varying concentrations of substrates.

| Substrate                | $K_m$ | $V_{max}$ |
|--------------------------|-------|----------|
| 11,12-LTA4               | 18    | 2380     |
| LTA4                     | 25    | 2080     |
| 14,15-LTA4               | 48    | 1450     |
| trans-β-Methylstyrene oxide | 833  | 2857     |

Table IV
Comparison of various leukotrienes and trans-β-methylstyrene oxide as substrates of cytosolic epoxide hydrolase.

| Substrate                          | $K_m$ | $V_{max}$ |
|------------------------------------|-------|----------|
| Chalcone oxide                     |       |          |
| Glycol formed                      |       |          |

Table V
Substrate specificity of guinea pig cytosolic epoxide hydrolase with 11,12-LTA4 and various xenobiotic epoxides

The enzyme activities were determined according to descriptions under "Experimental Procedures" at substrate concentrations described below. Chalcone oxide was added to a final concentration of 20 μM.

| Substrate                          | Chalcone oxide | Purified enzyme |
|------------------------------------|----------------|----------------|
| Glycol formed                      |                |                |
| Chalcone oxide                     |                |                |
| Added                              |                |                |
| n mole/min/mg protein              |                |                |
| % inhibition                        |                |                |

| Substrate                          | None | Added | % inhibition | None | Added | % inhibition |
|------------------------------------|------|-------|--------------|------|-------|--------------|
| 11,12-LTA4 (30 μM)                 | 1.16 | 0.18  | 84           | 1530 | 179  | 88           |
| Styrene oxide (2 mM)               | 45.0 | 35.1  | 22           | ND   |       |              |
| trans-β-Methylstyrene oxide (2 mM)| 7.56 | 1.52  | 80           | 3248 | 1113 | 66           |
| trans-β-Ethylstyrene oxide (2 mM)  | 9.90 | 2.02  | 80           | 2293 | 692  | 70           |
| trans-Stilbene oxide (0.5 mM)      | 0.72 | 0.17  | 76           | 643  | 193  | 70           |
| cis-Stilbene oxide (0.5 mM)        | 0.37 | 0.12  | 68           | 198  | 51   | 74           |

*The supernatant (105,000 × g, 60 min) was dialyzed against 10 mM Tris-HCl, pH 7.3, 1 mM Na2EDTA, 0.1 mM dithiothreitol.

*ND, not detectable, less than 0.01 nmol/min/mg protein.
(18-48 μM) were much lower than that for trans-β-methylstyrene oxide (833 μM), the best xenobiotic substrate among those examined in the present study (Table V). This finding indicates that the cytosolic epoxide hydrolase acts on endogenous substrates more preferably than on xenobiotic epoxides. It is of interest whether or not the previously reported xenobiotic epoxide hydrolases also catalyze the facile conversion of 11,12-LTA₄ to 11,12-diHETE.

In addition to the above enzymic product, at least seven dihydroxy acids were produced from 11,12-LTA₄. One of the important findings in the present study is isolation and structural identification of 12-HETE-δ-lactones formed from 11,12-LTA₄ by nonenzymic acid-catalyzed rearrangement. A possible mechanism of 12-HETE-δ-lactone formation is depicted in Fig. 5, Scheme 1. The 12-hydroxy intermediate was formed, and the carboxyl moiety could attack the carbonium ion at C₅ position. On the other hand, the 5-hydroxy intermediate was formed from LTA₄ (Fig. 5, Scheme 2), and the carboxyl moiety was unable to attack the C₅ position. This might explain the difference in acid-catalyzed reactions between LTA₄ (40) and 11,12-LTA₄. These lactones are useful indicators to demonstrate the formation of 11,12-LTA₄ from arachidonic acid.

In conclusion, 11,12-LTA₄ is nonenzymically converted to at least seven compounds (Fig. 5): two diastereomers of 12-HETE-δ-lactones (C-5, C-6), three stereoisomers of 11,12-diHETE (C-3, C-3', and C-4), and two diastereomers of (5,12S)-diHETE (C-1, C-2). (11R,12S)-Dihydroxy-5,14-cis-7,9-trans-eicosatetraenoic acid is synthesized by the action of the cytosolic epoxide hydrolase purified from the guinea pig liver (Fig. 5). Although the 12-lipoxygenase activity was reported in porcine liver (17), whether 11,12-LTA₄ serves as a substrate of the hepatic epoxide hydrolase in vivo remains unclear. It is possible to speculate that the enzyme is involved in the hydrolysis of 11,12-LTA₄, which has been transferred from the circulating leukocytes/platelets. An analogous transport of LTA₄ was reported from neutrophils to erythrocytes (48), to arterial endothelial cells (49) and platelets (50). The acquisition of structural information of all these compounds makes feasible analysis of the formation of 11,12-LTA₄ in vivo and of the biological significance of 12-lipoxygenase pathway.

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SUPPLEMENTARY MATERIAL

Saponification of 11,12-HETE by Protein from Several Sources

by

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EXPERIMENTAL PROCEDURES

MATERIALS. Commercial sources of reagents and materials were as follows: Methylene chloride, from Nacalai Tesque, Inc., Osaka, Japan; methanol, from Nacalai Tesque, Inc., Osaka, Japan; ethanol, from Kanto Chemicals (Tokyo); and 50% (v/v) ethanol, from Kanto Chemicals (Tokyo). 11,12-HETE was synthesized according to the procedures of Kii et al. [1986] and Corey and Barton [1982].

METHODS. Column chromatography was performed on silica gel (Merck, Darmstadt, Germany) and Sephadex G-10 (Pharmacia, Uppsala, Sweden) as indicated. The protein concentration was determined by the method of Lowry et al. [1951] with bovine serum albumin as standard.
HPLC. Structure of C-3

Twice the addition of methanal and HCl to adjust pH to 2.5. Products were extracted at -70°C. Fast atom bombardment mass spectrometer (FAB-MS) was performed for a 3-30 pmol FT MS spectrometer. The FAB spectrum from labeled spectrophotometry was performed using a 218-218 FT MS spectrometer (1950).

Analysis of epoxide hydrolase with various hemiolic acids and esters

Identification of the specific activity, the specific activity, the specific activity of the partially purified enzyme (after ammonium sulfate fractionation) and the specific activity of the partially purified enzyme in the guinea pig liver cytosol. The enzyme activity was determined with 77.12-LTA4 (1) and 11.12-LTA4 as a substrate. The enzyme activity was assayed at 37°C without any detectable loss of activity for 1 month.

Phosphoesterases: The activity of the enzyme from the guinea pig liver was 95.8 upon SDS-PAGE (Fig. 6). The activity of the enzyme was assayed with a superoxide 12. Adrenal gland

Superoxide dismutase, which was purified by reversed-phase chromatography (Superose, SP-5PW and 2nd Superose 12), which resulted in acquisition of a single peak at around 160 ml. The epoxide hydrolase activity toward styrene oxide and n-butylbenzene was assayed by a method described under "EXPERIMENTAL PROCEDURES."
Fig. 4 SDS-PAGE of the enzyme at different stages of purification. The gel was 0.8 mm thick and had a concentration of 12% acrylamide. Lane 1, cytosol (105,000 g supernatant); lane 2, ammonium sulfate fraction; lane 3, Q-Sepharose; lane 4, first Superose 12; lane 5, Phenyl-Sepharose; lane 6, SP-Sephadex); lane 7, second Superose 12. Mr of standard proteins are: 45,000, phosphorylase b; 67,500, bovine serum albumin; 13,500, ovalbumin; and 94,000, carbonic anhydrase.

| Molecular weight| Isoelectric point | N-terminal amino acid | Blockage |
|-----------------|-------------------|-----------------------|----------|
| 65,000          | 7.3               | Yes                   |          |

Table II. Molecular properties of guinea pig liver cytochrome c peroxidase.

a Determined by SDS-PAGE.
b Ala values were determined disregarding Trp and Cys.