Enzymatic Hydration of Leukotriene A₄

PURIFICATION AND CHARACTERIZATION OF A NOVEL EPOXIDE HYDROLASE FROM HUMAN ERYTHROCYTES*

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(Received for publication, November 8, 1984)

Human erythrocytes contained a soluble cytosolic epoxide hydrolase for stereospecific enzymatic hydration of leukotriene A₄ into leukotriene B₄. The enzyme was purified 1100-fold, to apparent electrophoretic homogeneity, by conventional DEAE-Sephacel® chromatography followed by high performance anion exchange and chromatofocusing procedures. Its characteristics include a molecular weight of 54,000 ± 1,000, an isoelectric point of 4.9 ± 0.2, a Kₐ apparent from 7 to 36 µM for enzymatic hydration of leukotriene A₄, and a pH optimum ranging from 7 to 8. The enzyme was partially inactivated by its initial exposure to leukotriene A₄. There was slow but detectable enzymatic hydration (pmol/min/mg) of certain arachidonic acid epoxides including (±)-14,15-epoxy-5,8,11-eicosatrienoic acid and (±)-11,12-epoxy-5,8,14-eicosatrienoic acid, but not others, including 5,6-epoxy-8,11,14-eicosatrienoic acid. Human erythrocyte epoxide hydrolase did not hydrate either styrene oxide or trans-stilbene oxide. In terms of its physical properties and substrate preference for leukotriene A₄, the erythrocyte enzyme differs from previously described versions of epoxide hydrolase. Human erythrocytes represent a novel source for an extrahaematopoietic, cytosolic epoxide hydrolase with a potential physiological role.

A prominent role for epoxide hydrolase is hepatic detoxification of xenobiotic, electrophilic oxiranes (1, 3). However, the significance of enzymatic hydration is not restricted to hepatic metabolism or to xenobiotic detoxification. Interactions between extrahaematopoietic sources of epoxide hydrolase and certain endogenous substrates are also biologically relevant. Leukotriene A₄ (LTA₄), a transient allylic epoxide of xenobiotic, electrophilic oxiranes certain endogenous substrates are also biologically relevant. Leukotriene B₄ (LTB₄), a chemokinetic, myotropic, dihydrodiol (6S,12R)-5,12-dihydroxy-6,14-cis-8,10-trans-eicosatrienoic acid) implicated in inflammatory and respiratory disorders (8-10). In the absence of epoxide hydrolase, spontaneous, nonenzymatic hydration of the unstable substrate (LTA₄) occurs. As a result, no LTB₄ is produced; instead, biologically reactive 5,12- and 5,6-dihydrodiol isomers (4, 11-13) are formed. Among those cells that can convert LTA₄ into LTB₄, human erythrocytes attract attention from two perspectives. First, in contrast to other cells, particularly leukocytes (6, 7, 14-17), erythrocytes have seldom been attributed any capacity for eicosanoid biosynthesis until recently (18, 19). Furthermore, erythrocytes have not been recognized as a source of extrahaematopoietic epoxide hydrolase activity for hydration of xenobiotic oxiranes (2).

In view of its unexpected presence and its uncertain role in human erythrocytes, we have purified and partially characterized an epoxide hydrolase that converts LTA₄ into LTB₄. This enzyme differs in several respects from previously identified forms of epoxide hydrolase (1-3, 20-24).

**Experimental Procedures**

**Materials**—Synthetic LTA₄, methyl ester (The Upjohn Co.) and [3H]LTA₄ methyl ester, 50 Ci/mmol (New England Nuclear), were purified prior to use (25). LTA₄ lithium salt was prepared as described (26). Synthetic leukotrienes (The Upjohn Co.) including LTB₄, trans-LTB₄, 6-trans-LTB₄, 6,12-epi-LTB₄, and 14,15-epoxy-5,7,9,11-eicosatrienoic acid were prepared and purified as described (4, 27). Regioisomeric monooxepoxide derivatives of arachidonic acid, (±)-5,6-EET, (±)-11,12-EET, and (±)-14,15-EET (The Upjohn Co.) were synthesized as described (28-30). Prostaglandin B₃, microcrystalline cellulose, α-cellulose, human hemoglobin (Sigma), α-bromoacetophenone, trans-styrene oxide, trans-stilbene oxide, trans-stilbene, and 2N, N′-dilisopropylpentylethylene (Aldrich) were used as received. Water, hexane, ethyl acetate, and methanol all distilled-in-glass (Burdick & Jackson Laboratories, Inc.), Sep-Pak™ C₈ cartridges (waters Associates, Inc.), Nucleosil® 50-5 silica columns and CIS reversed-phase columns, 250 x 6 x 4 mm (Rainin Instruments), were used for metabolite isolation and quantitation by high performance liquid chromatography. Centricon™ 50 ultrafiltration devices (Amicon) were used to concentrate dilute protein solutions. [3H]Styrene oxide (New England Nuclear) and [3H]trans-stilbene oxide, kindly provided by Dr. Bruce Hammock, University of California, Davis, were used as received.

**Instrumental Analysis**—A Lambda 5 UV/VIS recording spectrophotometer (Perkin-Elmer), a model 110A positive displacement pump (Altex Beckman), a Spectromonitor D@ variable wavelength detector (Milton Roy), and a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) were used for spectroscopy and quantitative or preparative chromatographic procedures.

**Enzyme Preparation**—Purified erythrocyte suspensions were prepared as described (31). After depletion of leukocytes (≤5 x 10⁶/ml) and platelets (≤5 x 10⁶/ml) by filtration through cellulose columns, the resulting suspension (50 ml) contained 2-3 x 10⁷ human eryth-
rocytes/ml. Suspended erythrocytes were centrifuged at 200 x g for 20 min, and the supernatant fluid was discarded. Erythrocytes were then lysed hypotonically by resuspension in sterile water (50 ml). After restoration of isotonic conditions, the cytosolic fraction was separated from the stroma by centrifugation at 100,000 x g for 1 h. The 100,000 x g supernatant was filtered through a 0.45-μM Milipore filter, adjusted to 4 liters of water, dialyzed against 0.01 M Tris, pH 8.0, for 24 h at 4°C. The cell pellet was washed three times by centrifugation and resuspension in 0.01 M Tris, pH 8.0. Crude lysate containing membranes and cytosol, the post 100,000 x g supernatant, and the post 100,000 x g cell pellet were incubated at 37°C with LTA₄ (10 μM) to determine the subcellular distribution of epoxide hydrolase in human erythrocytes.

**Determination of Enzyme Activity**—Erythrocyte epoxide hydrolase activity was determined by measuring the conversion of LTA₄ into its enzymatic hydration product, LTB₄. For routine assays [³H]LTA₄ (10 nmol) dissolved in tetrahydrofuran (2.5–5 μl) was added to solutions of cytochalasin (1.0 ml, 40–50 mg/ml protein) or to partially purified enzyme (1.0 ml, 2–3 mg/ml protein) in 0.01 M Tris, pH 8.0. After incubation for 1 min at 37°C, residual substrate was hydrolyzed by quenching with 9 ml of 0.9% NaCl (w/v), pH 5.5. Prostaglandin B₂ (1 μg) was added as an internal standard and the mixture was processed through a Sep-Pak C₁₆ cartridge. Absorbed material was removed by sequential elution with 10 ml each of water, hexane, and ethyl acetate (32). The ethyl acetate fraction contained >95% of the adsorbed radioactive material including LTB₄, prostaglandin B₂, and a nonenzymatic hydration product. After evaporation of the solvent, the LTB₄ content in the residue was quantitated by RP-HPLC (6, 33).

For enzyme kinetic experiments, the substrate concentration was varied from 10 to 100 μM by incubating enzyme (0.5 ml, 2–3 mg/ml protein) with 5–50 nmol of LTA₄ dissolved in 2.5–5 μl of tetrahydrofuran. For kinetics at 37°C, the enzyme reaction was terminated at 1 min by quenching with 5 ml of 0.9% NaCl, pH 5.5, to destroy unreacted substrate. Enzymatic kinetics were also determined at 25 and 4°C. At these temperatures, in some experiments the amount of intact LTA₄ was determined after a 1-min incubation to assure that it was present in sufficient excess. Intact LTA₄ was quantitated by RP-HPLC as previously described (26).

**DEAE Anion Exchange Chromatography**—After dialysis against 0.01 M Tris, pH 8.0, approximately 10–20 ml (40–50 mg/ml protein) of the 100,000 x g supernatant fluid was applied to a 2.5 x 20-cm DEAE-Sepharose (Pharmacia) anion exchange column equilibrated with 0.01 M Tris, pH 8.0. Nonbound material was removed by elution with 0.01 M Tris, pH 8.0. The material that bound to the anion exchanger including hemoglobin and epoxide hydrolase, was eluted at 0.5 ml/min with a linear gradient of 250 ml each of 0.01 M Tris, pH 8.0, and 0.01 M Tris, pH 8.0, containing 1 M NaCl. The absorbance at 415 and 280 nm was monitored in 10-ml fractions, and their protein content was determined spectrophotometrically (34). Fractions were concentrated 2-fold by ultrafiltration through Centricon 30 microconcentrators (Amicon) and assayed for epoxide hydrolase activity as described above. Active fractions were pooled and dialyzed against 0.01 M Tris, pH 8.0, for use in subsequent experiments.

**Substrate Specificity**—Certain epoxide metabolites of arachidonic acid (35, 36) and selected xenobiotic epoxides were also evaluated as substrates for human erythrocyte epoxide hydrolase. Enzymatic hydrolysis of [³H]trans-stilbene oxide and [³H]styrene oxide was measured as described (37, 38). Enzymatic hydrolysis of 5,6-EET, 11,12-EET, and 14,15-EET was determined by an adaptation of RP-HPLC procedures (35, 39). Samples (1.0 ml) of enzymatic reaction mixtures containing 5–300 nmol of substrate were quenched with 5% NaCl (4.0 ml), pH 5.5, and extracted with ethyl acetate (3 x 5 ml). The organic layers were evaporated under nitrogen, and residual 5,6-EET, 11,12-EET, or 14,15-EET was determined spectrophotometrically (34). Enzymatic hydration of 5,6-EET, 11,12-EET, or 14,15-EET and their corresponding hydration products were formed (Fig. 1).

**Partial Purification and Characterization**—Fractionation of dialyzed 100,000 x g supernatant fluid by anion exchange chromatography on DEAE-Sepharose revealed that the epoxide hydrolase activity was distinct from the major hemoglobin peak (Fig. 2). The specific activity of DEAE-purified enzyme increased 5-fold to 0.36 nmol of LTB₄/min/mg (Table 1).
Enzymatic hydration of LTA₄ by DEAE-purified enzyme was pH-dependent, with maximal formation of LTB₄, occurring eluted with methanol/water/acetic acid, partially purified enzyme transformed LTA₄ (10⁻⁴ M) into 0.72, 1.10, 0.88, 0.90, 0.76, and 0.44 nmol of LTB₄/ml, respectively.

Formation of LTB₄ was linearly related to the protein concentration in the reaction mixture. Addition of 10 μM LTA₄ to DEAE-purified enzyme preparations containing 0, 0.28, 0.56, 0.84, 1.1, 1.1, 1.4, 1.7, 2.2, 2.8, 5.6, and 8.4 mg of protein/ml yielded 0.15, 0.27, 0.36, 0.44, 0.57, 0.69, 0.98, 1.17, 2.49, and 3.72 nmol of LTB₄/ml, respectively.

According to Lineweaver-Burk analysis, the apparent KM for erythrocyte epoxide hydrolase ranged from 7 to 36 μM, and the Vₘₐₓ ranged from 0.29 to 1.43 nmol of LTB₄/ml/min for DEAE-purified enzyme obtained from three blood donors.

These values are based on LTB₄ formation after 1 min at 37°C. Under these conditions, the amount of intact, residual substrate may have declined considerably by accompanying, nonenzymatic hydration. Consequently, kinetics were also determined at 25 and 4°C. At these temperatures, intact substrate was still present in excess after a 1-min incubation. For instance, enzyme reaction mixtures contained (mean ± S.D., n = 5) 81 ± 7, 75 ± 6, and 60 ± 8% of the initially added LTA₄ intact, after incubation at 4°C for 1, 2, and 5 min, respectively. Enzyme reaction mixtures contained 52 ± 7% of the initially added LTA₄ intact, after incubation at 25°C for 1 min. At 25°C, the apparent KM ranged from 9 to 30 μM; at 4°C, KM apparent ranged from 19 to 36 μM.

Suicide Inactivation by LTA₄—At 25 and 4°C, LTB₄ formation was maximal within 2 min even though the enzyme reaction mixtures contained a sufficient excess of LTA₄ to sustain enzymatic hydration for a longer time. This suggested that epoxide hydrolase was inactivated by its reaction with LTA₄. This was substantiated by demonstrating that accumulation of LTB₄ was not proportional to successive, supplemental additions of LTA₄ to enzyme. For example, an initial incubation of DEAE-Sepharcel-purified enzyme (1.0 ml, 3 mg) with 10 nmol of LTA₄ produced 2.2 nmol of LTB₄/ml. After two successive additions of LTA₄ (10 nmol), at 2-min intervals, cumulative LTB₄ production increased to 2.4 and 3.0 nmol/ml, respectively. Therefore, LTB₄ production per incremental addition of LTA₄ corresponded to 2.2, 0.2, and 0.6 nmol of LTB₄/ml/10 nmol of LTA₄ or increases of 9 and 25% per respective addition. There was no evidence of product inhibition by LTB₄ (10 μM) or its nonenzymatic 5,12-dihydrodiol isomers (10–100 μM). Furthermore, when fresh enzyme (1.0 ml, 3 mg) was mixed with enzyme (1.0 ml, 3 mg) inacti-
vated by LTA₄, the fresh enzyme could synthesize LTB₄. Addition of LTA₄ (10 nmol) to such a mixture restored cumulative LTB₄ production from 1.3 to 2.9 nmol/ml. Activity associated with addition of fresh enzyme was consistent with suicide inactivation, but not product inhibition, since the incubation mixture contained LTB₄ and nonenzymatic hydration products derived from prior exposure to substrate.

**Substrate Specificity**—Several other epoxides were evaluated as substrates. There was slow, but detectable enzymatic hydration of certain epoxide metabolites of arachidonic acid such as 14,15-EET and 11,12-EET but not others, such as 5,6-EET. Enzymatic hydration of 14,15-EET in the 100,000 × g supernatant fluid transformed 14,15-EET (50 μM) and 11,12-EET (50 μM) into their corresponding vicinal diols at respective rates of 1.78 ± 0.57 pmol/min/mg (mean ± S.E., n = 6) and 0.59 ± 0.11 pmol/min/mg (n = 4). Initial hydration rates were linearly proportional to the protein content in the incubation mixtures. The plot of protein concentration (abscissa, 0-42 mg/ml) versus 14,15-EET hydration rate (ordinate, 0-10.1 nmol/h/mg) was linear with a correlation coefficient of 0.99 and a slope of 0.24 nmol/h/mg. For 11,12-EET, the plot was linear with a correlation coefficient of 0.99 and a slope of 0.031 nmol/h/mg. The initial hydration velocity was constant for at least 15 h. Nonenzymatic hydration in 0.01 M Tris, pH 8.0, at 37°C was negligible: 0.0029 and 0.0014 h⁻¹, respectively, for 14,15-EET and 11,12-EET. Enzymatic hydration of 5,6-EET (50 μM) was indistinguishable from its nonenzymatic hydration which occurred spontaneously at 0.33 ± 0.05 nmol/min/ml (mean ± S.D., n = 4) in 0.01 M Tris, pH 8.0, at 25°C. According to Lineweaver-Burk analysis, 14,15-EET had a Kᵣ apparent of 20 μM and Vₘₙₙ of 2.3 pmol/min/mg. Accurate determination of the apparent Kᵣ for 11,12-EET was not possible due to its slow hydration rate. At various stages of purification, there was no detectable enzymatic hydration of [³H]trans-stilbene oxide or [³H]styrene oxide, indicating that these common substrates for hepatic epoxide hydrolase (43) were poor substrates for erythrocyte epoxide hydrolase.

**Inhibition of LTB₄ Formation**—Some epoxides that were not substrates inhibited enzymatic hydration of LTA₄ in a dose-dependent manner. For instance, trans-stilbene oxide at 10, 100, and 1000 μM inhibited LTB₄ formation by 0, 51 ± 9, and 88 ± 10%, respectively. In contrast, trans-stilbene, chalcone, trans-1,2-dibenzylolethylene, chalcone oxide (44), 11,12-EET, and 14,15-EET were poor inhibitors even at concentrations exceeding 100 μM. Dithiothreitol (10 mM), p-bromophenacyl bromide (10-100 μM), iodoacetamide (100-250 μM), and iodoacetic acid (100-250 μM) did not affect enzymatic hydration.

**Purification to Electrophoretic Homogeneity**—The epoxide hydrolase present in DEAE-Sepharose fractions was purified to electrophoretic homogeneity by FPLC anion exchange chromatography on a Mono-Q (HR 5/5) column (Fig. 3) followed by FPLC chromatofocusing on a Mono-P (HR 5/20) column (Fig. 4). SDS-PAGE of the active Mono-P fraction contained a single band with a pI 4.9, a specific activity of 83.5 nmol/min/mg, and M₅₅, 54,000 ± 1,000 (Fig. 5). Table 1 summarizes the purification scheme.

**DISCUSSION**

An epoxide hydrolase involved in leukotriene metabolism has not been purified or characterized extensively. Consequently, it is uncertain to what extent it resembles or differs from multiple forms of the hepatic enzyme involved in xenobiotic metabolism. The latter have been characterized sufficiently to facilitate a comparison (1-3, 20-24). Our results indicate that human erythrocytes contain a distinct cytosolic epoxide hydrolase for efficient enzymatic hydration of LTA₄. This enzyme differs in several respects from known microsomal or cytosolic forms of hepatic epoxide hydrolase. Its molecular weight (M₅₅, 54,000) is similar to values reported for microsomal (M₅₅, 50,000-53,000) or cytosolic (M₅₅, 58,000) hepatic enzyme (22, 24, 45, 46). However, its stability, with no evident inactivation after 7 h at 37°C, exceeds that reported for cytosolic (25-40% inactivation after 7 h at 37°C) (23) or microsomal (10% inactivation after 24 h at 25°C) hepatic enzymes (45). Its substrate specificity for LTA₄ and its inability to catalyze hydration of either styrene oxide or trans-stilbene oxide distinguish it from any known form of hepatic epoxide hydrolase. The latter efficiently hydrate one and often
both of these xenobiotic substrates (1–3, 37, 38). Furthermore, human erythrocyte cytosolic epoxide hydrolase hydrates 14,15-EET and 11,12-EET at rates (μmol/min/mg) that differ by 104-fold from hydration rates (μmol/min/mg) reported for hepatic cytosolic enzyme (39). Such differences in substrate specificity have been useful for categorizing different hepatic epoxide hydrolases (43). Conversion of LTA4 to LTB4 by erythrocyte epoxide hydrolase was unaffected by several compounds that can inhibit the corresponding hepatic enzyme (44). The pH optimum (7.0–8.0) and pl (4.9 ± 0.2) for the erythrocyte enzyme concur with values reported for hepatic, cytosolic enzyme (pH optimum 7.0 (43), pl 5.1–6.1 (24)), but differ from the pH optimum (8.9–9.4) for hepatic microsomal enzyme (45). It is noteworthy that human erythrocytes also resemble in some, but not all, respects an LTA4 hydrolase (50).

Values for $K_m$ and $V_{max}$ can be derived from Lineweaver-Burk analyses; however, it is necessary to stress that enzymatic hydration of LTA4 conforms imperfectly to Michaelis-Menten kinetics for two reasons. First, spontaneous, nonenzymatic hydration of LTA4 accompanies its enzymatic hydration. At 37 °C, rapid depletion of LTA4 by both processes reduces the interval during which enzymatic hydration velocity remains constant. At 4 and 25 °C, this problem is less significant. Second, suicide inactivation by LTA4 also limits the duration of constant enzymatic velocity. Since LTB4 formation was maximal within 1 min, the values for initial reaction rate used in Lineweaver-Burk plots were based on LTB4 formation in that span. The initial rate of enzymatic hydration may have been somewhat higher; therefore, values for $V_{max}$ are a conservative estimate. The values obtained for $K_m$ (7–36 μM), are plausible from a physiological perspective, and they were independent of temperature in the kinetic experiments. Stable substrates, such as 14,15-EET or 14,15-EETE with negligible rates of spontaneous hydrolysis, with no evident suicide inactivation effects, and with persistent initial hydration velocity, conformed closely to Michaelis-Menten kinetics.

It is interesting to note that several enzymatic processes involved in biosynthesis and metabolism of eicosanoids are similar to those involved in activation or detoxification of xenobiotics. In addition to the epoxide hydrolase system, glutathione S-transferase may conjugate either eicosanoids or xenobiotics, and the cyclooxygenase-peroxidase enzyme complex may also catalyze cooxidation of xenobiotics (48, 49). The electrophilic and hydrophobic nature of many eicosanoids or xenobiotics probably accounts for this uniformity. Purification and biochemical characterization of functionally discrete enzymes is warranted to determine if pharmacological goals, such as selective modulation of eicosanoid biosynthesis, are attainable without corresponding impairment of fundamental detoxification processes.

In summary, human erythrocytes are a novel extrahepatic source for epoxide hydrolase. In terms of either eicosanoid biosynthesis or xenobiotic metabolism, there was no cellular metabolic precedent to anticipate its presence in erythrocytes, until recently (18). Its capacity for conversion of a naturally occurring epoxide into a biologically active dihydrodiol, LTB4, suggests that this may be an important function, under certain circumstances. Further investigation is warranted in this regard. In view of the losses during isolation and purification and the established multiplicity of hepatic forms of epoxide hydrolase, we cannot exclude that additional forms of erythrocyte epoxide hydrolase may exist. The erythrocyte enzyme resembles in some, but not all, respects an LTA4 hydrolase isolated from human leukocytes (50).

Acknowledgments—We thank D. Morton for synthesis of epoxides, Bruce Hammock for [3H]trans-stilbene oxide, and Jan Zelenock for secretarial assistance.

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