Characterization of leukotriene A_4 synthase from murine mast cells: Evidence for its identity to arachidonate 5-lipoxygenase

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ABSTRACT Leukotriene A_4 synthase was purified from the cytosolic fraction of murine mast cells. The enzyme converted 5-hydroperoxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid (5-HPETE) to leukotriene A_4. This unstable product was identified by demonstration of two epimers of 6-*trans*-leukotriene B_4, methanol trapping, as well as further transformation to leukotriene B_4 by leukotriene A_4 hydrolase. Leukotriene A_4 synthase stereospecifically eliminated the D-hydrogen at C-10 (pro-*R*) in the synthesis of leukotriene A_4 when incubated with [10D-^{3}H;3-^{14}C]5-HPETE. The purified enzyme also exhibited 5-lipoxygenase activity toward arachidonic acid and 8-lipoxygenase activity towards 8,11,14-*cis*-icosatrienoic acid. All of these activities required Ca^{2+} and ATP for their maximal velocities. The effects of heat treatment and of several lipoxygenase inhibitors on these enzyme activities as well as coelution in various chromatographic systems strongly suggest that lipoxygenase and leukotriene A_4 synthase activities reside in the same enzyme molecule.

The leukotrienes (LTs) (1) constitute a family of arachidonic acid metabolites involved not only in inflammatory or pathological processes but also in neuroendocrine functions. Formation of LTs is initiated by the 5-lipoxygenation of arachidonic acid to yield (5*S*)-5-hydroperoxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid (5-HPETE).

Lipoxygenase catalysis involves stereospecific abstraction of hydrogen, and in this case the D-hydrogen at C-7 of arachidonic acid is preferentially removed (2). In the following conversion [5-HPETE to (5*S*)-5-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid (LTA₄)] the D-hydrogen at C-10 is also removed (3, 4). Thus, there are mechanistic similarities between these two reactions, and recently we demonstrated that a single enzyme (from potato tubers) catalyzed both reactions by virtue of its dual lipoxygenase activities (5-lipoxygenase and 8-lipoxygenase) (5). In this paper we present data showing that also a single enzyme from a mastocytoma cell line converts arachidonic acid to 5-HPETE and sequentially to LTA₄.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Arachidonic acid (56 mCi/mmol; 1 Ci = 37 GBq) was purchased from Amersham. [10D-^{3}H;3-^{14}C]Arachidonic acid was a kind gift from A. Panossian at the Karolinska Institutet (4). Arachidonic acid and 8,11,14-*cis*-icosatrienoic acid (C20:3) were from Nu Chek Prep; linoleic acid was from Sigma. 5-HPETE, [1-^{14}C]5-HPETE, and [10D-^{3}H;3-^{14}C]5-HPETE were prepared by using potato lipoxygenase (5). 13-Hydroxylinoleic acid was synthesized by using soybean lipoxygenase (Sigma). AA-861 [2,3,5-

trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone] (6) was kindly provided by Takeda Pharmaceutical (Osaka, Japan); 5,6-methano-LTA₄ (7), (5*S*,12*R*)-5,12-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid (LTB₄), and prostaglandin B₂ (PGB₂) were from Ono Pharmaceutical (Osaka, Japan). The FPLC system equipped with columns Mono Q (HR 5/5) and Superose TM 12 (HR 10/30) was a product of Pharmacia. A hydroxyapatite column (Bio-Gel HPHT 0.78 × 10 cm, Bio-Rad) was used in connection with the FPLC system.

Assay of LTA₄ Synthase. *HPLC method.* The standard assay mixture contained 0.1 M Tris·HCl buffer (pH 7.5), 2 mM CaCl₂, 2 mM ATP, the microsomal protein from PB-3c cells (1 mg/ml), and enzyme in a total volume of 0.5 ml. The microsomal fraction, an activator of 5-lipoxygenase (8), also stimulated the LTA₄ synthase reaction by 10–30%. After preincubation (30°C, 2 min) the incubation (5 min) was initiated by addition of 5-HPETE (50 μM, 25 nmol in 3 μl of ethanol). The reaction was terminated by addition of 1 ml of methanol (−20°C) with 800 pmol of PGB₂ as internal standard. Following centrifugation, the supernatant was evaporated to dryness and reconstituted in 0.1 ml of solvent A [acetonitrile/methanol/H₂O/acetic acid, 3:1:3:0.006 (vol/vol), containing 0.05% EDTA]. HPLC analysis was performed with a TSK gel ODS column 80-TM (4.6 × 150 mm, Toyo Soda) eluted with solvent A (1 ml/min), and the absorbance at 270 nm was monitored. Enzyme activity was estimated from the ratio of peak areas (LTA₄-derived products to PGB₂). When C20:3 was included in the reaction mixture as an inhibitor, [1-^{14}C]5-HPETE (25 nmol, 86,000 cpm) was used as a substrate, and the radioactivity corresponding to hydrolysis products of LTA₄ was measured. The recoveries of LTs and PGB₂ were constant (95–97%).

Spectrophotometric methods. The incubation mixture was as described above except that ATP was omitted. After addition of 5-HPETE, the increase in the absorbance at 275 nm was followed. This method was less appropriate, since the omission of ATP diminished the activity by 30–50%.

Assay of Lipoxygenases. The standard assay mixture (0.2 ml; composition, see above) was preincubated (2 min, 30°C) and arachidonic acid (80 μM, 16 nmol in 2 μl of ethanol) was added. 15-Hydroperoxyicosatetraenoic acid (5 μM) was added to the assay mixture together with the arachidonic acid. The incubation (5 min) was terminated with 0.5 ml of methanol (−20°C) containing 3 nmol of 13-hydroxylinoleic acid (internal standard). Following centrifugation, the supernatant was evaporated to dryness and reconstituted in 0.2 ml of methanol. For HPLC analysis, the ODS column was eluted

Abbreviations: LT, leukotriene; 5-HPETE, (5*S*)-5-hydroperoxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid; LTA₄, (5*S*)-5-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid; LTB₄, (5*S*,12*R*)-5,12-dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid; 5,12-DiHETE, (5*S*,12*S*)-5,12-dihydroxy-6,10-*trans*-8,14-*cis*-icosatetraenoic acid; PG, prostaglandin; C20:3, 8,11,14-*cis*-icosatrienoic acid.
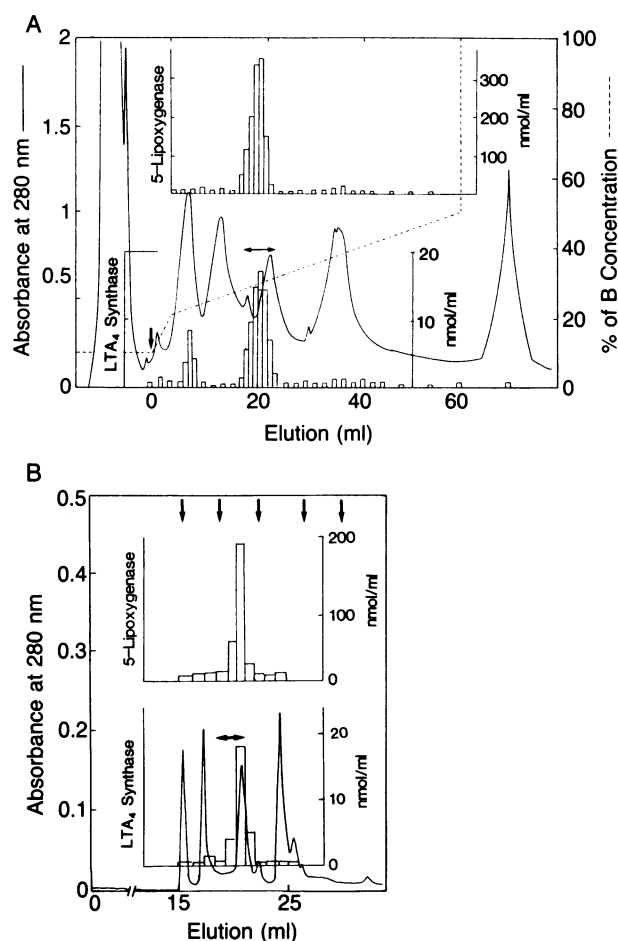
FIG. 1. (A) Anion-exchange chromatography (Mono Q) of ammonium sulfate fraction (35–60%) from PB-3c cells. The solid line indicates the absorbance at 280 nm; the dashed line denotes the concentration of buffer (B) (100% = 1 M KCl). Active fractions between arrows were combined for further purification. (B) Gel filtration on Superose TM 12. Vertical arrows indicate the positions of markers [blue dextran (M_r 2,000,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), soybean trypsin inhibitor (M_r 22,000), and cytochrome c (M_r 13,000); from left to right]. The active fractions between horizontal arrows were combined and are referred to as the purified enzyme.

with methanol/H_2O/acetic acid, 75:25:0.01 (vol/vol) (1 ml/min), and the absorbance at 234 nm was monitored. Lipoxygenase activity was determined from the ratio of peak areas [(5S)-5-hydroxy-6-*trans*-8,11,14-*cis*-icosatetraenoic acid (+ 5-HPETE) to the internal standard; recoveries >88%]. C20:3 8-lipoxygenase activity was measured essentially as described above, except that arachidonic acid was replaced by C20:3. In all assays, 1 unit of activity was defined as that producing 1 nmol of product per 5 min at 30°C.

Cells. The interleukin-3 (IL-3)-producing murine WEHI-3b cell line (9) was donated by B. M. Stadler of the Institute of

Table 1. Subcellular distribution of LTA_4 synthase in PB-3c cells

Sample	Protein, mg	Total activity, units	Specific activity, units/mg
Supernatant			
800 × g	120	336	2.8
10,000 × g	85	382	4.5
105,000 × g	78	250	3.2
105,000 × g pellet	12	—	<0.02

Homogenates of PB-3c cells (5 × 10⁸ cells in buffer A, 10 ml) were subjected to centrifugation as indicated. The pellet was resuspended in 1 ml of buffer A.

Clinical Immunology, University of Bern, Switzerland. The mouse bone marrow-derived mast cell line (PB-3c) (10) was generously provided by J.-F. Conscience of Friedrich Miescher Institute, Basel, Switzerland, and was further subcloned for more satisfactory IL-3-dependent proliferation. WEHI-3b-conditioned medium was prepared as described (9). The subclone of PB-3c was maintained by regular passages in 50 vol of fresh RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 10% WEHI-3b-conditioned medium at intervals of 3–4 days. Cells at confluence were harvested and used as the enzyme source. After washing, cells (2.5 × 10⁹) were suspended in 50 ml of 20 mM potassium phosphate buffer, pH 7.4/0.1 M NaCl/2 mM EDTA/0.5 mM dithiothreitol (buffer A) saturated with N_2 gas.

Purification of LTA_4 Synthase from PB-3c Cells. All procedures were carried out at 0–4°C, except for FPLC chromatographies (room temperature). Buffers were saturated with N_2 gas and degassed thoroughly before use. Protein concentrations were determined according to Bradford (11) with bovine serum albumin as standard. The cell suspension was homogenized by sonication (Branson B-12, output 4, 5 × 10 sec). After centrifugation (10,000 × g, 15 min) the supernatant (referred to as crude extracts) was subjected to ammonium sulfate fractionation. The precipitate between 35% and 60% was reconstituted in 7.5 ml of 20 mM Tris·HCl, pH 8/1 mM EDTA/0.5 mM dithiothreitol/20% glycerol (buffer B) and applied to three PD-10 columns (Pharmacia) equilibrated with the same buffer. The pooled macromolecule fractions (10.5 ml) were concentrated to 3 ml (ultrafiltration, PM 10, Amicon). After filtration (Millipore, 0.22 μm) the sample (3 × 1 ml) was applied to the Mono Q column equilibrated with buffer B. The enzyme was eluted by increasing the ionic strength as shown in Fig. 1A. Active fractions were combined and concentrated, and the buffer was changed (PD-10) to 5 mM potassium phosphate buffer, pH 7.6/1 mM EDTA/1 mM dithiothreitol/20% glycerol (buffer C).

This sample was applied to the hydroxyapatite column equilibrated with buffer C. The enzyme was eluted by increasing the concentration of potassium phosphate (up to 0.3 M) at a flow rate of 0.4 ml/min. The pool of active fractions was concentrated to 0.4 ml by using a Centricon 10 (Amicon).
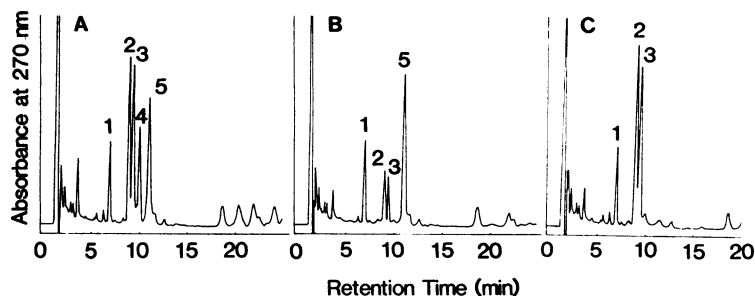

FIG. 2. HPLC analyses of products formed in incubations with 80 μM 5-HPETE. Reactions were carried out under standard assay conditions. (A) Ten thousand × g supernatant fraction of PB-3c cell homogenates (0.8 mg of protein). (B) As in A except that Ca^{2+} and ATP were omitted. (C) The purified enzyme (4.3 μg). Peak 1, PGB_2; peak 2, 6-*trans*-LTB_4; peak 3, 12-*epi*-6-*trans*-LTB_4; peak 4, LTB_4; peak 5, 5,12-DiHETE.

Table 2. Purification of LTA$_4$ synthase from PB-3c cells

| Purification | Protein, mg | Specific activity, units/mg | | Ratio* | Yield,[†] % |
		LTA$_4$ synthase	5-Lipoxy-genase		
Crude extracts	889	3.1	29	0.11	100
Ammonium sulfate	255	8.5	52	0.16	79
Mono Q	10.1	59	664	0.09	22
HPHT (Bio-Rad)	1.0	179	1730	0.10	6.5
Superose TM 12	0.05	310	3870	0.08	0.6

*The ratio was expressed as the LTA$_4$ synthase activity/5-lipoxygenase activity determined under standard assay conditions.
[†]Yield was calculated on the basis of the LTA$_4$ synthase activity.

The final purification step was gel filtration on Superose TM 12 eluted with 20 mM potassium phosphate buffer, pH 7.4/0.2 M NaCl/1 mM EDTA/0.5 mM dithiothreitol/20% glycerol (buffer D, 0.3 ml/min). Active fractions as indicated by the arrows were combined and are referred to as the purified enzyme (Fig. 1B).

RESULTS

Enzymatic Synthesis of LTA$_4$. When 5-HPETE was incubated with crude extracts of cell homogenates, as shown in Fig. 2A, 6-trans-LTB$_4$, 12-epi-6-trans-LTB$_4$, LTB$_4$, and (5S,12S)-5,12-dihydroxy-6,10-trans-8,14-cis-icosatetraenoic acid (5,12-DiHETE) were formed. Ca^{2+} and ATP were required for the synthesis of the former three compounds (peaks 2–4), whereas the formation of 5,12-DiHETE was only slightly affected (Fig. 2B). When the purified enzyme was used instead of crude extracts, only the isomers of 6-trans-LTB$_4$ were observed. These results indicate that the cell homogenates contained LTA$_4$ synthase, LTA$_4$ hydrolase, and 12-lipoxygenase; the latter two enzymes were removed during purification. The existence of an epoxide intermediate (LTA$_4$) was demonstrated as follows. First, 1-min incubations of 5-HPETE (25 nmol) with enzyme (0.1 mg of protein, 180 units/mg) were terminated by addition of 10 vol of acidic methanol (12). The products were extracted with diethyl ether and subjected to reverse-phase HPLC in solvent A. A pair of peaks (18.2 and 19.8 min) showed UV spectra with maxima at 258, 269, and 280 nm. The compounds were converted to methyl ester trimethylsilyl ether derivatives and analyzed by GC/MS. Both compounds appeared with a C value of 24.3 (2% OV-1), showing ions at m/z 421 (M-15), 404, 389, 293, 234, 203, 171, 159 (base peak), and 129, in fair agreement with reported data for the epimers at C-12 of (5S)-5-hydroxy-12-O-methyl-6-trans-LTB$_4$ (12). Second,

the addition of partially purified LTA$_4$ hydrolase (human leukocytes) to the standard reaction mixture produced LTB$_4$. These results indicate the presence of LTA$_4$ in the synthesis of 6-trans-LTB$_4$ and its 12-epimer.

Copurification of LTA$_4$ Synthase and Arachidonate 5-Lipoxygenase: Some Properties of the Enzyme. Upon subcellular fractionation, LTA$_4$ synthase was recovered in the cytosol fraction (Table 1). The purification procedure gave a protein of about 85% purity, as judged by NaDod-SO$_4$/PAGE, and the yield was 0.6%. LTA$_4$ synthase and 5-lipoxygenase copurified, and the ratio of the two activities was 0.08–0.1 after Mono Q column chromatography (Table 2). The diverging ratio in the crude preparations may reflect nonenzymatic formation of LTA$_4$ (13). A major band (M_r 75,000) always appeared on the NaDodSO$_4$ gel in parallel with the enzyme activity, whereas the M_r was estimated to be 50,000–55,000 by gel filtration (Superose TM 12). The enzyme was unstable especially after Mono Q column chromatography, even in the presence of glycerol, dithiothreitol, and EDTA, and under anaerobic conditions, which all partially stabilized the enzyme. Addition of microsomal protein from the PB-3c cells back to the purified enzyme (or to the 105,000 × g supernatant of the homogenate, Table 1) stimulated enzyme activities, as shown for 5-lipoxygenase from human leukocytes. In contrast, there was no similar stimulatory effect of the protein nonadsorbing to the Mono Q column (8, 14).

Synthesis of LTA$_4$ proceeded almost linearly for the first 2–3 min (Fig. 3A), was dependent of the amount of enzyme (up to 10 μg), and was diminished by boiling for 3 min (Fig. 3B). The enzyme required Ca^{2+} for full activity; the half-effective dose was ≈0.1 mM (Fig. 3C). Omission of ATP from the reaction mixture decreased the activity by almost 50%. When cells were treated with 2 μM Ca ionophore (A23187, Calbiochem) for 5 min in Tyrode solution and washed with buffer A prior to homogenization, the enzyme activity of the
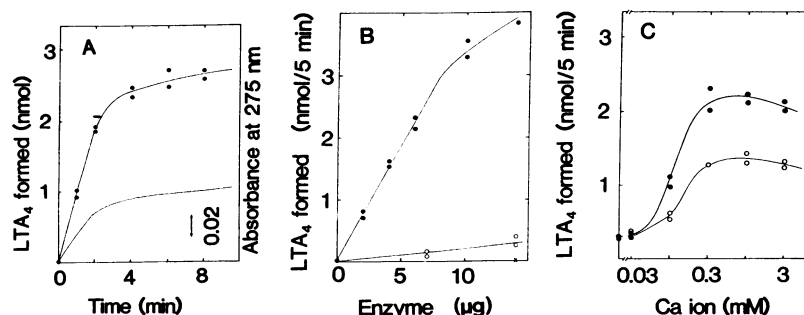

FIG. 3. (A) Time course of LTA$_4$ synthase determined by HPLC assay (●) and spectrophotometric assay. Approximately 8 μg of enzyme (180 units/mg, after HPHT column) was incubated with 5-HPETE under standard assay conditions. The solid line indicates the increase in the absorbance at 275 nm with the same amount of enzyme. (B) Effect of enzyme amount (0–15 μg) either in the presence (●) or absence (○) of 2 mM Ca^{2+} with 2 mM ATP. LTA$_4$ was not formed with an enzyme boiled for 3 min (×). (C) Requirement of Ca^{2+} for LTA$_4$ synthase either in the presence (●) or absence (○) of ATP.

10,000 × *g* supernatant was lowered by 25–30% compared to the control. The inclusion of EDTA during all steps of the enzyme purification was in fact required for obtaining reproducible enzyme activities.

When the purified enzyme (2.4 μg) was incubated with 80 μM arachidonic acid (with Ca^{2+} and ATP), about 7.4 nmol of 5-HPETE and 2.1 nmol of LTA_4 were produced. The enzyme produced about 2.5 times more LTA_4 when incubated with arachidonic acid than with 5-HPETE (50 μM), although less 5-HPETE was formed in the arachidonate incubation.

Also under standard assay conditions, the purified enzyme (2.4 μg) acted on C20:3 to give 0.8 nmol of 8-hydroperoxy-9,11,14-icosatrienoic acid (identified by HPLC, UV spectroscopy, and GC/MS; data not shown).

Heat Inactivation and Inhibitors. When the enzyme was treated for 3 min at various temperatures, 5-lipoxygenase and LTA_4 synthase activities decreased concomitantly (Fig. 4). In the crude extracts, however, about 25% of the LTA_4 synthase activity was heat-resistant (13). The lipoxygenase inhibitors AA-861 (Fig. 4) and 5,6-methano-LTA_4 inhibited both enzyme activities. IC_{50} values of the latter compound for LTA_4 synthase and 5-lipoxygenase activities were 25 and 18 μM, respectively. Finally, C20:3, another substrate of the enzyme, competitively inhibited (K_i = 0.15 mM) the arachidonate 5-lipoxygenation and inhibited the formation of LTA_4 from 5-HPETE in a dose-dependent manner (data not shown).

Incubation of the Purified Enzyme with [10D-^{3}H;3-^{14}C]5-HPETE. The purified enzyme as well as crude extracts of cell homogenates were incubated with 10 μM [10D-^{3}H;3-^{14}C]5-HPETE (20,000 cpm for ^{3}H and 51,000 cpm for ^{14}C). Metabolites were isolated by HPLC and the radioactivity ratio was determined for each compound. As shown in Table 3, LTA_4 (6-*trans*-LTB_4, 12-*epi*-6-*trans*-LTB_4) synthesized with the purified enzyme, and LTB_4 (formed only in crude extracts) lost >92% of the D (pro-*R*)-hydrogen when compared to the ^{3}H/^{14}C ratio of the precursor 5-HPETE. On the other hand, 5,12-DiHETE retained the D-hydrogen at C-10. The apparent partial retention of 10D (pro-*R*) in the formation of LTA_4 by crude homogenates is explained by hemoproteins catalyzing synthesis of LTA_4 and isomeric compounds by a different mechanism (13). Interestingly, however, the ^{3}H/^{14}C ratio of LTB_4 formed in the crude extracts was significantly lower, probably reflecting the preference of LTA_4 hydrolase for enzymatically formed LTA_4, with the correct stereochemistry.
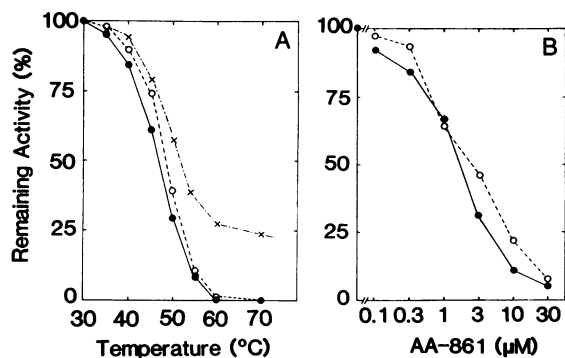

FIG. 4.   Effect of heat treatment and inhibition on LTA_4 synthase (●) and 5-lipoxygenase (○) activities of the purified enzyme (180 units/mg). (*A*) Samples of 5 μg were treated at different temperatures for 3 min and cooled on ice. Also in crude extracts (0.8 mg) the effect on LTA_4 synthase was determined (×). (*B*) Samples were preincubated (2 min) with different concentrations of AA-861.

Table 3. ^{3}H/^{14}C ratios of products from incubations with [10D-^{3}H;3-^{14}C]5-HPETE

Compound	^{3}H/^{14}C			
	Purified enzyme		Crude extracts	
6-*trans*-LTB_4	2	4	33	22
12-*epi*-6-*trans*-LTB_4	3	5	32	28
LTB_4	—		8	5
5,12-DiHETE	—		98	106

Experiments in duplicate were carried out with crude extracts (3.1 units/mg) and the purified enzyme (310 units/mg) from PB-3c cells. The ^{3}H/^{14}C ratio of the precursor 5-HPETE (0.392) was defined as 100%.

DISCUSSION

Biologically active LTs are synthesized by way of the unstable intermediate LTA_4. Several studies have shown the enzymatic nature of LTA_4 formation from 5-HPETE (3, 4, 12) and indicated that the enzyme(s) involved were cytosolic (15). Here, we describe a cytosolic, Ca^{2+}-requiring LTA_4 synthase from murine mast cells. The PB-3c cell line was chosen because of the prominent conversion of arachidonic acid by the 5-lipoxygenase pathway. In our hands, the specific activity of LTA_4 synthase in these cells was about 6 times higher than in RBL cells and 2.5 times higher than in human leukocytes (data to be published elsewhere).

The LTA_4 synthase from PB-3c cells was purified to about 85% homogeneity. Many properties were similar to those of mammalian arachidonate 5-lipoxygenases (8, 14–19): the molecular weight (75,000), the acidic isoelectric point, and the requirements for Ca^{2+} and ATP. In addition, there are mechanistic similarities between the reactions catalyzed by 5-lipoxygenase and LTA_4 synthase (5). Thus, several criteria suggested the identity of LTA_4 synthase with arachidonate 5-lipoxygenase. This concept was further supported by the following findings: (*i*) both enzyme activities copurified (Table 2) and coeluted in three principally different FPLC chromatographies; (*ii*) both enzyme activities responded practically identically to heat treatment and to inhibitory agents (Fig. 4); (*iii*) C20:3 was a substrate for the enzyme (oxygenated at C-8), which inhibited LTA_4 synthase and 5-lipoxygenase activities; (*iv*) when the purified enzyme was incubated with [10D-^{3}H:3-^{14}C]5-HPETE, a selective elimination of the 10D-hydrogen was observed, in agreement with data on the mechanism of LTA_4 synthesis in intact cells (3, 4).

The data presented here indicate that in murine mast cells, as was demonstrated recently for LTA_4 formation in human leukocytes (20), a single enzyme catalyzes 5-lipoxygenation and further conversion to LTA_4. These findings are of considerable interest in connection with physiologic and therapeutic control of LT formation.

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