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Purification, Characterization, and Structural Properties of a Single Protein from Rat Basophilic Leukemia (RBL-1) Cells Possessing 5-Lipoxygenase and Leukotriene A_4 Synthetase Activities

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SUMMARY

Arachidonate 5-lipoxygenase of rat basophilic leukemia (RBL-1) cells was purified more than 1000-fold by gel filtration and anion exchange protein-high performance liquid chromatography (HPLC). Physical properties of the purified 5-lipoxygenase such as molecular weight (74,000–76,000), N-terminal sequence (30 amino acids), and amino acid composition were determined. The purified enzyme converted [^{14}C]arachidonic acid at 20° to [^{14}C] 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and to [^{14}C]dihydroxyeicosatetraenoic acids (diHETEs). Utilizing [^{14}C] 5(*S*)HPETE as substrate, the purified enzyme also converted the hydroperoxy acid to [^{14}C]diHETES. The [^{14}C]diHETE reaction products were identified primarily (>80% of recovered radioactivity) as the nonenzymatic hydrolysis products of leukotriene A_4 (i.e., 6-*trans*-leukotriene B_4 and 12-epi-6-*trans*-leukotriene B_4) by reverse phase HPLC, scanning spectrophotometry, and gas chromatography-mass spectrometry. The bioconversion of [^{14}C] arachidonate and [^{14}C]5(S)HPETE to reaction products by the purified enzyme was dependent on the presence of both Ca^{2+} and ATP. The enzymatic activities were inhibited in a similar manner by the lipoxygenase inhibitors nordihydroguaiaretic acid, diphenyldisulfide, and SK&F 86002. The data provide evidence that RBL-1 cell 5-lipoxygenase and leukotriene A_4 synthetase activities reside on a single monomeric protein with a free N-terminus and that they possess similar biochemical characteristics.

The enzyme 5-lipoxygenase catalyzes the oxygenation of free arachidonic acid to form 5-HPETE which is the first step in the biosynthesis of the leukotrienes. The leukotrienes are a well characterized family of potent, biologically active agents that have an important role in the pathophysiology of a variety of inflammatory and allergic responses (1). In addition, 5-lipoxygenase is believed to be involved in the biosynthesis of the lipoxins through the oxidation of 15-HPETE (2).

5-Lipoxygenase has been enriched to varying degrees from guinea pig leukocytes (3), RBL-1 cells (4), and human (5) leukocytes and purified extensively from RBL (2H3) cells (6), human leukocytes (7), pig leukocytes (8), and murine mast cells (9). The enzyme displays lag and inactivation kinetics (6, 7) and is dependent upon Ca^{2+} and ATP for maximal activity (7–9).

An additional activity demonstrated for potato 5-lipoxygenase (10) and reticulocyte 15-lipoxygenase (11) is the further enzymatic conversion of the hydroperoxy acid (i.e., 5-HPETE or 15-HPETE) to a conjugated triene epoxide (i.e., 5,6- or 14,15-LTA_4). Evidence of LTA_4 synthetase activity for the highly enriched 5-lipoxygenase from pig leukocyte (8), human leukocyte (12), and murine mast cell (9) has also been reported. Thus, a key step in the formation of LTB_4 and the peptidoleukotrienes (i.e., formation of LTA_4) has been hypothesized to be a property of the single enzyme, 5-lipoxygenase.

The intent of the present study was to purify 5-lipoxygenase to homogeneity from a single mammalian cell source and to determine its ability to convert arachidonate and 5-HPETE to LTA_4. Thus, the RBL-1 cell 5-lipoxygenase was purified to homogeneity, the reaction products of the 5-lipoxygenase/LTA_4 synthetase activities were identified, structural properties of the purified protein were determined, and biochemical properties of the two enzyme activities were characterized.

ABBREVIATIONS: RBL, rat basophilic leukemia; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; RP-HPLC, reverse phase high performance liquid chromatography; GC/MS, gas chromatography-mass spectrometry; HPETE, hydroxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; diHETE, dihydroxyeicosatetraenoic acid; SK&F 86002, 6-(4-fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-*b*]thiazole; NDGA, nordihydroguaiaretic acid; EDTA, ethylenediaminetetraacetate.

Experimental Procedures

Materials. [1-^{14}C]Arachidonate (58 mCi/mmol) was from Amersham (Arlington Heights, IL). Biosynthetic 5(*S*)HPETE and [1-^{14}C] 5(*S*)HPETE (8.8 mCi/mmol) were from Biomol Inc. (Philadelphia, PA). NDGA, phosphatidylcholine (type III-E), ATP, and arachidonic acid were from Sigma (St. Louis, MO). Diphenyldisulfide was from Fluka Chemical Co. (Hauppauge, NY). Enzyme grade ethylene glycol, fluoraldehyde reagent diluent, BRIG 35, 2-mercaptoethanol, *o*-phthalaldehyde, sequanal grade hydrochloric acid, and sequanal grade methanol were from Pierce Chemical Co. (Rockford, IL). Ammonium sulfate, ultra pure grade, was from Schwarz-Mann (Cambridge, MA). Molecular weight standards and electrophoresis reagents were from Bio-Rad (Rockville Centre, NY). Synthetic SK&F 86002, LTB_4, epi-LTB_4, *trans*-epi-LTB_4 and *trans*-LTB_4 were generously provided by Dr. Paul Bender (Department of Medicinal Chemistry, Smith Kline and French, Laboratories). All other chemicals and reagents were of the highest grades available from commercial sources.

Cell culture and supernatant preparation. RBL-1 cells from the American Type Culture Collection (Rockville, MD) were grown in spinner culture in Eagle's essential medium supplemented with 10% fetal calf serum (Hazelton Laboratories, Dutchland, PA). The cells ($5–10 \times 10^9$) were collected by centrifugation at $2,000 \times g$ for 20 min and washed twice with buffer A containing 50 mM $NaPO_4$ (pH 7.0) and 1 mM EDTA. The cells were resuspended in buffer A at 6.5×10^7 cells/ml and lysed by nitrogen cavitation at 800 psi for 10 min. The cell lysate was centrifuged at $10,000 \times g$ for 10 min and the resulting supernatant was brought to 5% ethylene glycol and centrifuged at $105,000 \times g$ for 60 min. The high speed supernatant was frozen in liquid nitrogen and stored at −70° until use.

5-Lipoxygenase purification. The frozen RBL-1 supernatants (90–120 ml) were thawed and brought to 25% saturation with 100% ammonium sulfate (pH 7.1) containing 100 μg/ml sonicated phosphatidylcholine. The mixture was stirred for 1 hr at 4° and centrifuged at $10,000 \times g$ for 10 min. The supernatants were pooled and brought to 60% saturation with 100% ammonium sulfate (pH 7.1) containing 100 μg/ml sonicated phosphatidylcholine. The mixture was stirred for 1 hr at 4° and centrifuged as described above. The pellets were resuspended in buffer B containing 25 mM bis-Tris (pH 7.1), 1 mM EDTA, 50 mM NaCl, 5% ethylene glycol, and 100 μg/ml sonicated phosphatidylcholine. The resuspended pellets were desalted through a PD-10 column (Pharmacia, Piscataway, NJ) and injected into a TSK-4000 gel filtration protein HPLC column preequilibrated with buffer B containing 20 μg/ml sonicated phosphatidylcholine at a flow rate of 2 ml/min. The gel filtration fractions with the highest 5-lipoxygenase activity (70–90 ml) were pooled and injected onto a TSK 5PW-DEAE anion exchange column preequilibrated at 4° with buffer B at a flow rate of 3 ml/min. The column was washed with buffer B at 3 ml/min for 15 min. The 5-lipoxygenase activity was eluted from the DEAE column with a nonlinear step gradient of 50–400 mM NaCl in buffer B at 4 ml/min. The DEAE fractions (30–40 ml) were pooled, brought to a concentration of 100 μg/ml sonicated phosphtidylcholine, and dialyzed at 4° against 2 liters of buffer B for 1 hr. The dialyzed sample was injected onto a Mono Q 10/10 column (Pharmacia, Piscataway, NJ) preequilibrated at 4° with buffer B at a flow rate of 3 ml/min. The Mono Q column was washed with buffer B for 15 min. The 5-lipoxygenase activity was eluted at 4 ml/min with a nonlinear step gradient of 50–400 mM NaCl in buffer B. The purified 5-lipoxygenase was frozen in liquid nitrogen and stored at −70° until use. The 5-lipoxygenase and LTA_4 synthetase activities were stable for 3–4 weeks at −70° with less than 20% loss of activity. Protein concentrations were determined by the method of Bradford (13) using bovine serum albumin as standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by modification of the method described by Laemmli (14) under reducing conditions.

Enzyme assay, HPLC analyses, UV-visible spectrophotometry, and radiochemical detection. Aliquots of RBL-1 supernatants or purified fractions were assayed either in buffer A containing 5% ethylene glycol (supernatant) or buffer B (purified fractions). All assays were performed at 20° in a final volume of 200 μl containing either arachidonate (100,000 dpm/assay tube of [^{14}C]arachidonate at 20,000 dpm/nmol specific activity) for 5-lipoxygenase or 5(*S*)HPETE (15,000 dpm/assay tube of [^{14}C]5(*S*)HPETE at 3,500 dpm/nmol specific activity) for LTA_4 synthetase. The reaction mixtures were incubated either in the presence or absence of 2 mM Ca^{2+} and/or ATP either for 3 min (5-lipoxygenase) or 15 min (LTA_4 synthetase) and deproteinized with 2 volumes (0.4 ml) of acetone. The reaction products were extracted (>95% of all radioactivity) into the organic phase once with 4 volumes (0.8 ml) and once with 2 volumes (0.4 ml) of ethyl acetate. A 1.5-ml aliquot of the upper organic phase was removed and evaporated to dryness under argon. The evaporated material was resuspended into 60 μl of methanol for RP-HPLC analyses.

The samples were analyzed by RP-HPLC using a 4.6×100 mm C_{18} column (Brownlee Laboratories, Santa Clara, CA) with gradient elution of acetonitrile:H_2O:H_3PO_4 of 500:500:0.05 (v/v) to 730:270:0.05 for 7 min and 1000:0:0 for 3 min at a flow rate of 2 ml/min. The separated reaction products were monitored for absorbance at 235 and 270 nm and analyzed by scanning spectrophotometry from 190 to 400 nm using a Hewlett-Packard (Palo Alto, CA) 1040A diode-array scanning spectrophotometer. The utilization of [^{14}C]arachidonate and [^{14}C]5-HPETE and the formation of ^{14}C-reaction products were quantitated using a Ramona-D radiochemical detector equipped with a 400-μl yttrium-packed flow cell (IN-US Service Corp., Fairfield, NJ). Enzyme activities were quantitated by integration of preset regions-of-interest for ^{14}C-substrates (i.e., [^{14}C]arachidonate and [^{14}C]5-HPETE and for ^{14}C-reaction products (e.g., [^{14}C]5-HPETE or [^{14}C]5,12-diHETEs). The integrated values of radioactivity were directly proportional to dpm values determined separately by scintillation spectrometry. Thus, the radioactivity in each preset region-of-interest was quantitated either in terms of nmol of product formed or substrate utilized.

Reaction product isolation and identification. Aliquots of Mono Q purified 5-lipoxygenase were assayed in buffer B containing 2 mM Ca^{2+} and 2 mM ATP at 20° in a total volume of 1 ml. The reaction mixtures were incubated either with 25 μM [^{14}C]arachidonate for 20 min or 5 μM [^{14}C]5-HPETE for 60 min and then deproteinized and extracted as described above. Extracts from several incubations were combined and resuspended in 0.8 ml of 30% acetonitrile in 50 mM ammonium acetate. Individual products were separated by RP-HPLC on a 10×250 mm Ultrasphere column (Beckman Instruments, Berkeley, CA) by gradient elution with acetonitrile and aqueous acetic acid (1%). Following a 400-μl injection, the reaction products were eluted in the following manner at a flow rate of 4.3 ml/min: acetonitrile was held isocratically at 41.4% for 36 min, increased linearly to 90% over 2 min, and held at 90% for 5 min. The eluate (0.2 ml/fraction) was collected for 7 min following a 31.5-min delay. Fractions containing products of interest were evaporated under reduced pressure and converted to their methyl esters with ethereal diazomethane. The products were subjected to thin layer chromatography (LK60 Linear-K silica gel, ether:ethyl acetate, 98:2). The plates were scraped, the products were extracted two times with 1 ml of ether, and the extracted samples were evaporated under nitrogen.

Trimethylsilyl ethers of the two lipoxygenase products were prepared by reaction with 20 μl of TRISIL-Z [(*N*,*O*-bis-trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane in pyridine (Suppelco, Bellefonte, PA)] for 30 min at 60°. Electron impact mass spectra were obtained on a Finnigan 4500 GC/MS (Finnigan, San Jose, CA) equipped with a fused silica capillary column (10 m × 0.32 mm) coated with a 0.25-μm-thickness film of DB-5 (J & W Scientific, Rancho Cordova, CA) and an injector maintained at 240°. Following injection, the oven was held at 50° for 2 min, increased ballistically (approximately 30°/min) to 220° and increased at 4°/min to 300°.

Amino acid analyses. The Mono Q fractions containing the highest 5-lipoxygenase activity were pooled (4–6 ml, 20–30 μg/ml) and dialyzed against 20 liters of 0.05% SDS for 24 hr. The dialyzed sample was

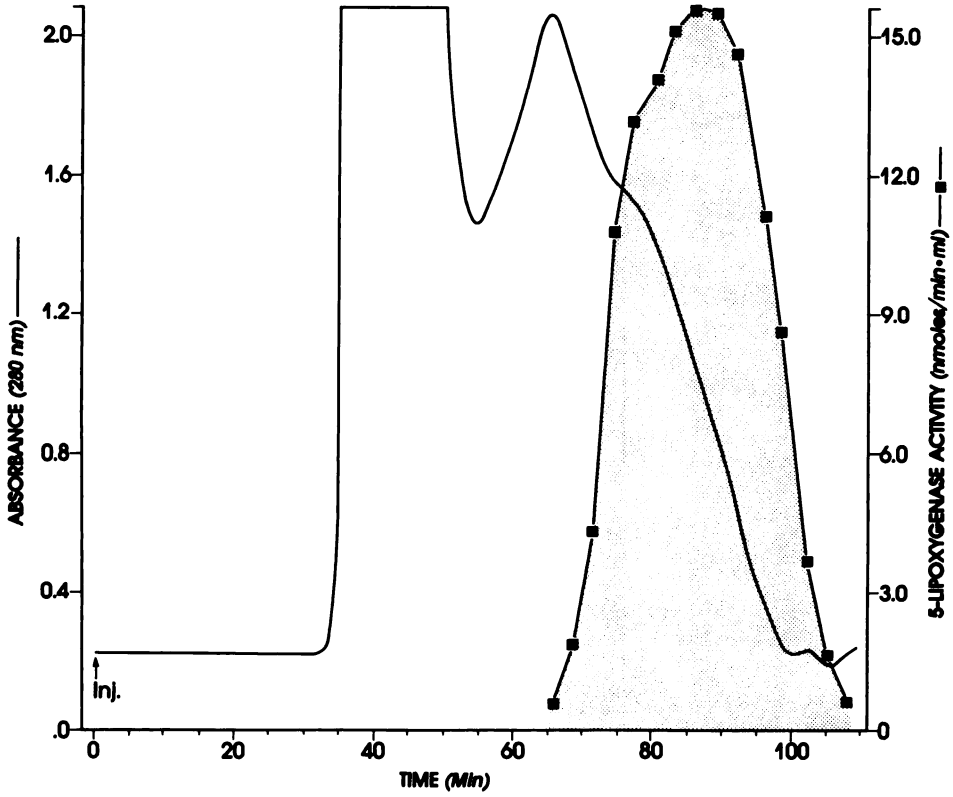

Fig. 1. Fractionation of RBL-1 5-lipoxygenase by gel filtration chromatography. The 25–60% ammonium sulfate fraction (310 mg in 19 ml) was injected at a flow rate of 2 ml/min onto a 2.15 × 60 cm TSK-4000 column preequilibrated with buffer B containing 20 μg/ml sonicated phosphatidylcholine. Fractions (6 ml) were collected and 50-μl aliquots were assayed for 5-lipoxygenase activity (*shaded area*) in the presence of 2 mM Ca^{2+} and ATP for 3 min at 20°. The reaction mixtures were deproteinized and extracted and the reaction products were separated by RP-HPLC as described in Experimental Procedures. The chromatogram is a representative example of 24 separate experiments.

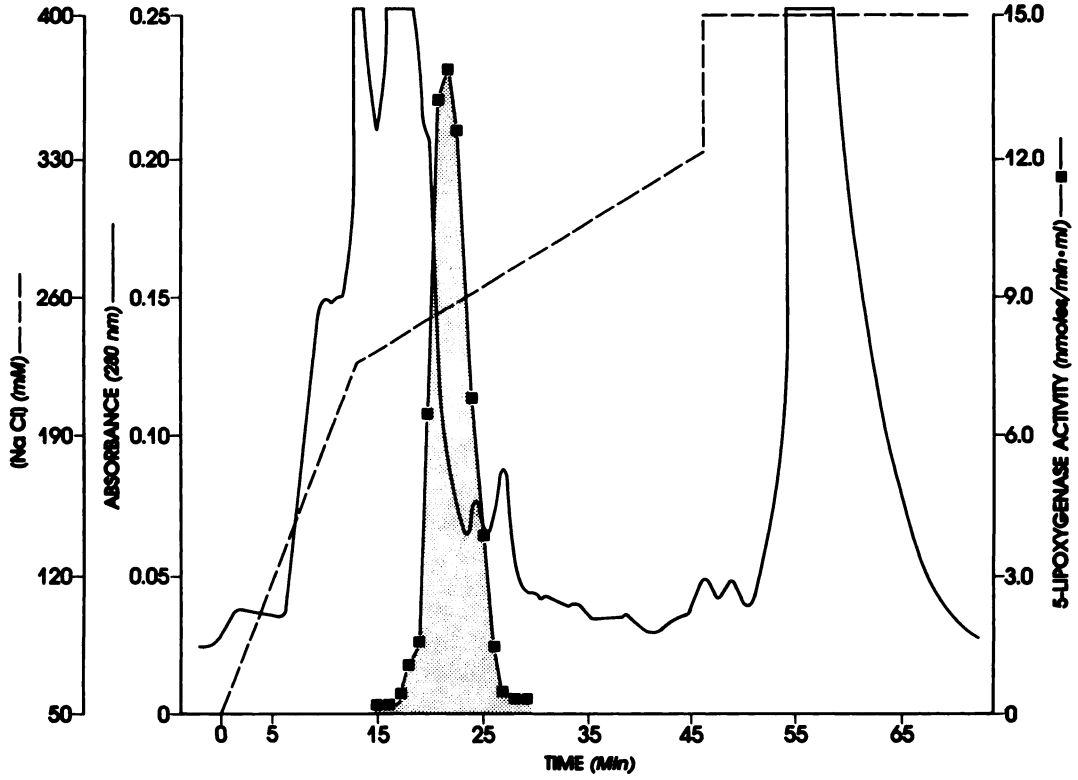

Fig. 2. Fractionation of RBL-1 5-lipoxygenase by weak anion exchange chromatography. Pooled gel filtration fractions (50 mg, 85 ml) were injected at a flow rate of 2 ml/min onto a 2.15 × 15 cm TSK DEAE-5PW column preequilibrated with buffer B. The 5-lipoxygenase activity (*shaded area*) was eluted into 6-ml fractions with a 50–400 mM NaCl gradient as indicated at a flow rate of 4 ml/min. The enzyme assay, reaction product extraction, and separation by RP-HPLC were determined as described in Experimental Procedures. The chromatogram is a representative example of 18 separate experiments.

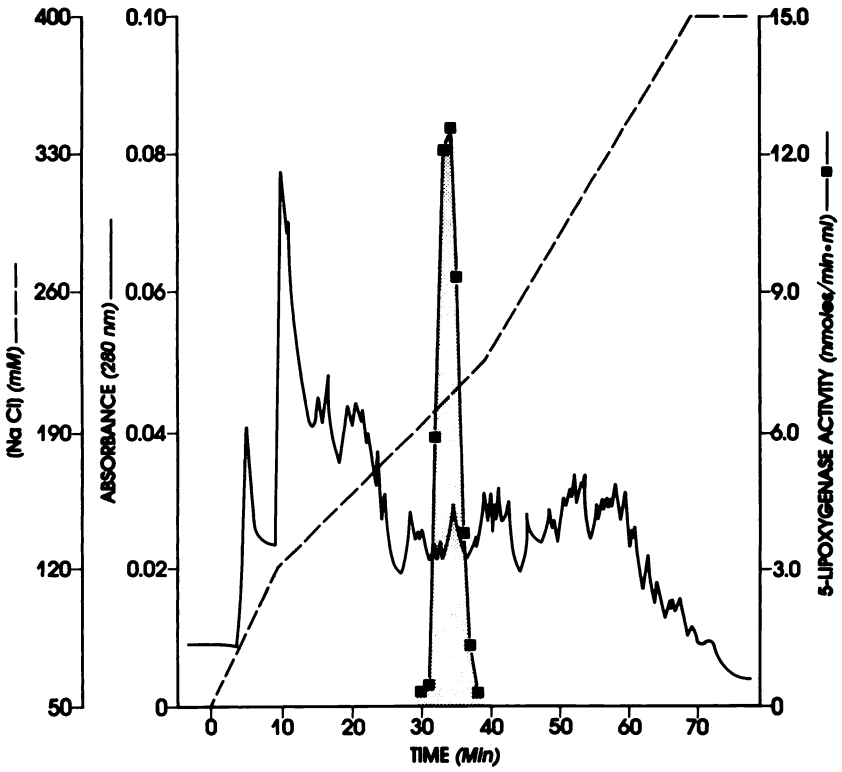

Fig. 3. Fractionation of RBL-1 5-lipoxygenase by strong anion exchange chromatography. The pooled (3 mg in 36 ml) DEAE fractions were dialyzed for 1 hr at 4° into 2 liters of buffer B containing 100 μg/ml sonicated phosphatidylcholine. The dialyzed sample was injected at a flow rate of 2 ml/min onto a Mono Q 10/10 column preequilibrated with buffer B. The 5-lipoxygenase activity (*shaded area*) was eluted with a 50–400 mM NaCl gradient as indicated at a flow rate of 4 ml/min. Aliquots of 4-ml fractions were assayed and the enzyme activity was determined as described in Experimental Procedures. The chromatogram is a representative example of 12 separate experiments.

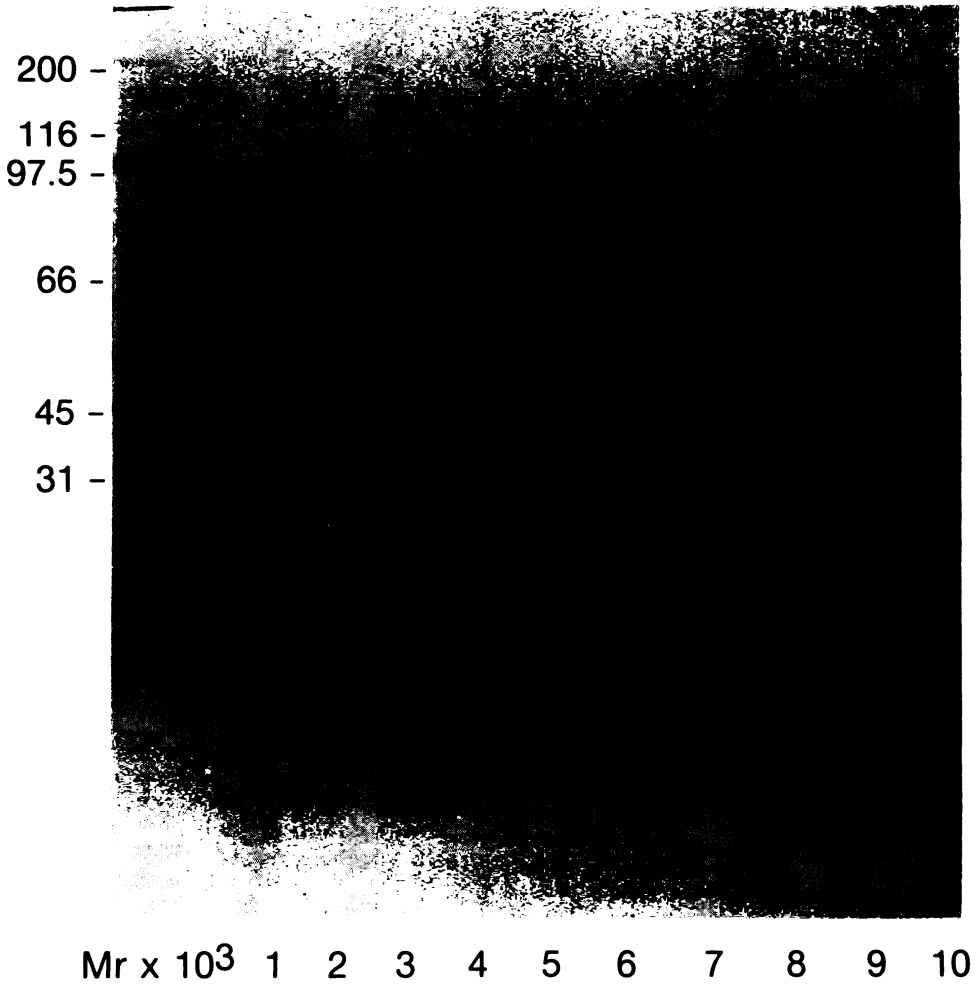

Fig. 4. Sodium dodecyl sulfate-polyacrylamide (12.5%) gel electrophoresis of RBL-1 5-lipoxygenase at various steps of purification. The molecular weight markers used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). Aliquots were taken from the following fractions: *1*, 0.25 mg of RBL-1 supernatant; *2*, 0.2 mg of 25–60% ammonium sulfate precipitate; *3*, 50 μg of TSK-4000 gel filtration fraction; *4*, 20 μg of DEAE-5PW fraction, *5*, 5 μg of Mono Q 10/10 fraction 34 of enzyme preparation A; *6–9*, 100-μl aliquots from Mono Q 10/10 fractions 32, 33, 34, and 35, respectively, of enzyme preparation B; and *10*, 5 μg of fraction 34 from enzyme preparation C. The gel is a representative example of five separate experiments.

TABLE 1

Purification of 5-lipoxygenase from RBL-1 cells

Aliquots of each fraction were assayed for 5-lipoxygenase activity at 20° for 30 sec in the presence of 25 μM [^{14}C]arachidonate, 2 mM $CaCl_2$, 2 mM ATP, 1 mM EDTA, 50 mM NaCl, 25 mM bis-Tris (pH 7.1), and 5% ethylene glycol. The reaction mixtures were extracted and enzyme activities were determined as described in Experimental Procedures.

Sample	Total protein	Total activity	Specific activity	-Fold purification	Yield
	mg	*nmol/min*	*nmol/mg-min*		%
High speed supernatant	479.3	1040.1	2.17	1	100
25–60% Precipitate	313.4	816.5	2.61	1.2	78.5
TSK gel filtration	49.4	675.0	13.7	6.3	64.9
DEAE 5-PW ion exchange	2.3	305.8	132.9	61.3	29.4
Mono Q 10/10 ion exchange	0.067	148.5	2217	1021	14.3

TABLE 2

Structural analyses of purified RBL-1 5-lipoxygenase

Enzyme purification, sample preparation, and protein analyses were performed as described in Experimental Procedures. The data for amino acid composition (A) are representative values of four separate experiments. The amino acids cysteine, proline, and tryptophan were not determined by the analyses used. Results are given as mol percent utilizing estimates of cysteine, proline, and tryptophan as 10% and assuming M_r = 75,000. The N-terminal amino acid sequence (B) represents data from four separate enzyme preparations. Two of the enzyme preparations are represented as lane 5 and lane 10 in Fig. 4. In each experiment, the amino acid sequence was the same.

A. Composition

Residue	Mol %	Residues/molecule
Asx	11.1	75
Glx	10.6	75
Ser	8.2	53
His	1.2	11
Gly	11.9	85
Thr	5.9	43
Arg	3.3	21
Ala	11.1	75
Tyr	4.0	32
Met	2.1	11
Val	6.5	43
Phe	3.9	32
Ile	5.4	43
Leu	9.4	64
Lys	3.8	21

B. N-terminal amino acid sequence

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pro	Ser	Tyr	Thr	Val	Thr	Val	Ala	Thr	Gly	Ser	Gln	Trp	Phe	Ala

16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gly	Thr	Asp	Asp	Tyr	Ile	Tyr	Leu	Ser	Leu	Ile	Gly	Glu	Ala	Gly

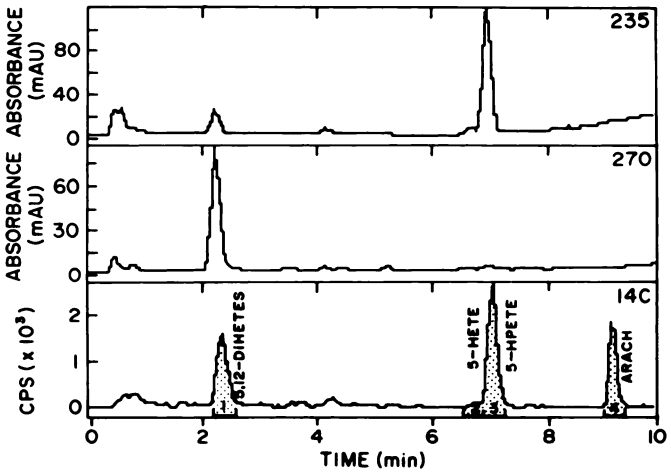

Fig. 5. RP-HPLC chromatograms of ^{14}C-reaction products produced from [^{14}C]arachidonate by purified 5-lipoxygenase. A 250-μl aliquot (10 μg) of fraction 34 from enzyme preparation C (see *lane 10*, Fig. 4) was incubated in the presence of 2 mM Ca^{2+}, 2 mM ATP, 25 mM bis-Tris (pH 7.1), 10% ethylene glycol, 100 μg/ml sonicated phosphatidylcholine, and 25 μM [^{14}C]arachidonate for 30 min at 20° in a total volume of 1 ml. The reaction products were extracted as described in Experimental Procedures and the evaporated material was resuspended in 0.25 ml of methanol. A 50-μl aliquot was removed for RP-HPLC analyses and the remainder was evaporated under argon and combined with other samples for GC/MS analyses (see Fig. 8). The *top* and *middle panels* are the absorbances at 235 and 270 nm, respectively, and the *bottom panel* is radiochemical (^{14}C) detection. The chromatograms are a representative example of four separate experiments.

reduced in volume to 0.4 ml with a Centricon-30 concentrator (Amicon, Danver, MA) and stored at 4° until use.

For hydrolyses, the protein samples were aliquoted into small glass vials and evaporated under reduced pressure using either a Savant Speed Vac concentrator or a Waters Picotag work station. The dried protein samples were added to 0.2 ml of constant boiling HCl in a Waters reaction vessel which was evacuated under reduced pressure and flushed with prepurified nitrogen three times after the addition of the HCl and protein sample. The protein sample was heated at 105° for 20 hr. After hydrolysis, the samples were solubilized in 0.1 ml of HPLC buffer A containing 20 mM sodium acetate (pH 5.9) and 2.5% tetrahydrofuran. A 10-μl sample was injected onto a 150 × 4.6 mm ultrasphere ODS column (Beckman) to react with a prior injection of 50 μl of *o*-phthalaldehyde derivatization reagent containing 50% fluoraldehyde reagent diluent (Pierce Chemical Co.), 0.3% BRIG 35, 0.2% 2-mercaptoethanol, and 2% *o*-phthaladehyde. The sample was allowed to react on the column for 2 min at room temperature and amino acids were eluted with a linear gradient of 20–80% methanol for 16 min at a flow rate of 1.5 ml/min.

Automated Edman degradation was performed on a modified Beckman 890 M sequencer utilizing the PPMMS sequencing program. Prior to the application of the sample to the sequencer, 1 mg of Polybrene (Aldrich, Milwaukee, WI) was applied to the spinning cup and one cycle without conversion was performed. Phthalaldehyde-derivatized amino acids were eluted at 50° from a 250 × 4.6 mm ultrasphere ODS column (Beckman) with a trifluoroacetic acid-acetate gradient system essentially as described previously (15).

Results

Purification and structural analyses of 5-lipoxygenase. The 5-lipoxygenase from RBL-1 cells has been localized previously (16) in the high speed supernatant (105,000 × *g* for 60 min) and is presumed to be a cytosolic enzyme. In the present studies, the high speed supernatant was routinely prepared from 5–10 × 10^9 RBL-1 cells grown in continuous spinner culture. The high speed supernatants, 70–90 ml, were frozen in liquid N_2 and stored at −70°. The 5-lipoxygenase activity in the supernatant was stable indefinitely under these conditions.

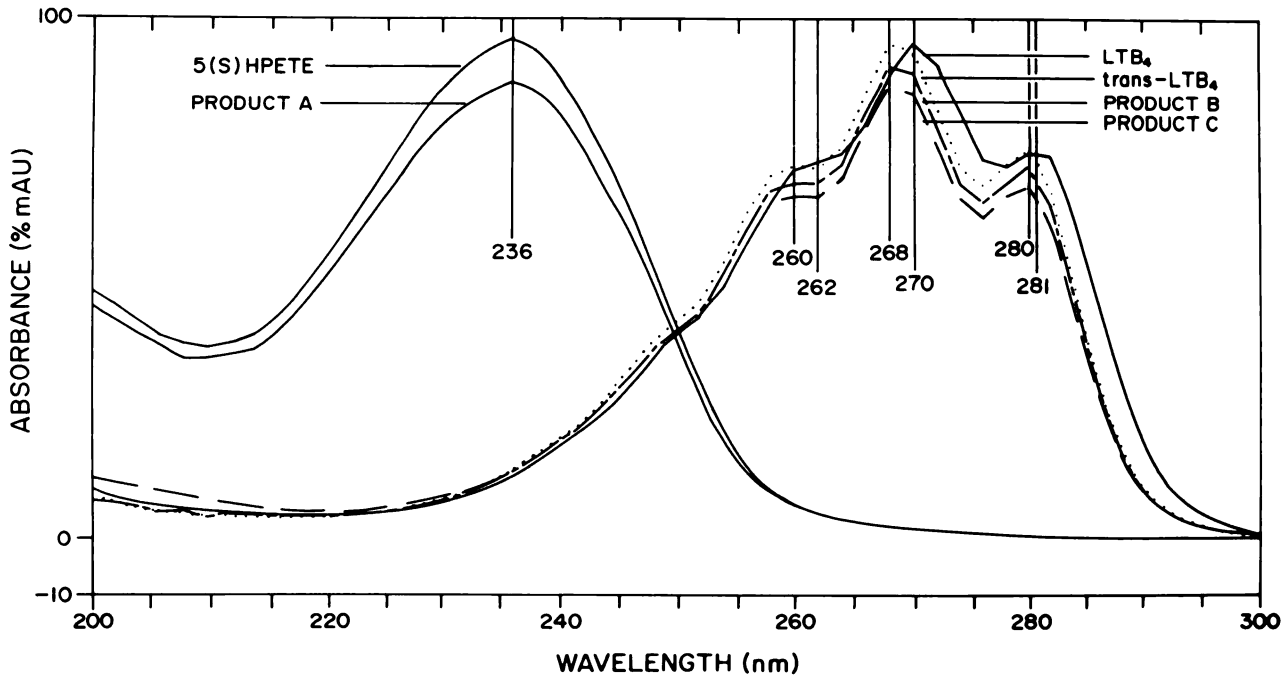

Fig. 6. Scanning spectra of ^{14}C-reaction products utilizing either [^{14}C]arachidonate or [^{14}C]5-HPETE as substrate. The scans were obtained for the ^{14}C-reaction products separated by RP-HPLC as described in Fig. 5 for [^{14}C]arachidonate and in Fig. 8 for [^{14}C]5-HPETE. Scanning spectra from 200 to 300 nm for each analysis were obtained as described in Experimental Procedures. *Products A* and *B* were reaction products extracted from the incubation of [^{14}C]arachidonate with the purified enzyme as described in Fig. 5 and had retention times corresponding to those of synthetic 5(*S*)HPETE and *trans*-LTB$_4$, respectively. *Product C* was extracted from the incubation of [^{14}C]5-HPETE as described in Fig. 8 and had a retention time corresponding to that of *trans*-LTB$_4$. The reference spectra for synthetic 5(*S*)HPETE, LTB$_4$, and *trans*-LTB$_4$ were obtained from separate RP-HPLC analyses. The spectra are representative examples of four separate experiments.

The enzyme activity was predominantly localized in the 25–60% ammonium sulfate fraction. A greater amount of activity was reproducibly recovered when 100 μg/ml sonicated phosphatidylcholine was present during the precipitation and resuspension of the ammonium sulfate fraction. The ammonium sulfate fraction was injected onto a TSK 4000sw gel filtration protein-HPLC column (Fig. 1). As observed for the ammonium sulfate precipitation, preequilibration of the column with buffer containing sonicated phosphatidylcholine (10–50 μg/ml) facilitated the reproducible recovery of 5-lipoxygenase activity. The 5-lipoxygenase activity eluted in a molecular weight range of 50,000–80,000, indicating that in the absence of Ca^{2+} and ATP the enzyme eluted in a native monomeric form.

The active gel filtration fractions were injected onto a TSK DEAE-5PW anion-exchange column (Fig. 2) and eluted at an NaCl concentration range of 240–260 mM. The purification step provided a 55- to 65-fold enrichment of 5-lipoxygenase activity and, more importantly, separated it from apparent peroxidase activity as determined by the absence of 5-HETE in the reaction product profile (results not shown, $N = 5$). After brief dialysis, the DEAE fractions were injected onto a Mono Q 10/10 protein-HPLC anion exchange column and the 5-lipoxygenase activity was eluted (Fig. 3) at an NaCl concentration of 200–230 mM. The fractions with peak enzyme activity contained a single protein of molecular weight 72,000–75,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). The molecular weight is similar to that of 5-lipoxygenase purified from pig leukocytes (8) and RBL-2H3 cells (6).

The purification of 5-lipoxygenase in the present report is a significant improvement over that in previous reports (7–9) for several reasons. The purification scheme reproducibly provided a stable and homogeneous preparation of 5-lipoxygenase with a purification of greater than 1000-fold and 10–15% yield (Table 1). The specific activity of 2.2 μmol/mg/min is substantially higher and indicates that stimulatory cofactors (7, 9, 12), in addition to Ca^{2+} and ATP, are not required for maximal enzymatic activity. The 5-lipoxygenase was stable for several weeks at −70° when stored in the presence of 1 mM EDTA, 15% ethylene glycol, and 100 μg/ml sonicated phosphatidylcholine.

The purified enzyme preparations shown in Fig. 4 were analyzed for amino acid composition and N-terminal amino acid sequence. Although cysteine, proline, and tryptophan were not determined in the composition, the values in Table 2 are a good estimate of the composition. The composition was obtained from enzyme samples that were also sequenced which verified homogeneity. The hydrophobicity ratio[1] of 5-lipoxygenase was 1.3, indicating a peripheral, non-membrane and cytosolic protein. A higher content of Asx and Glx compared to Lys, Arg, and His suggests that the 5-lipoxygenase is a relatively acidic protein which will bind to anionic exchange columns (i.e., DEAE-5PW and Mono-Q) at pH 7.1.

The N-terminal sequence of 5-lipoxygenase was determined reproducibly and with excellent yield. The data indicate that the enzyme preparations contain a homogeneous protein with a free N-terminus. With the exception of proline (at the N-terminus), the 5-lipoxygenase has no sequence homology with

[1] The hydrophobicity ratio is defined as the amino acid mole percent ratio of:

$$\frac{\text{Lys + Arg + Asx + Glx}}{\text{Ile + Tyr + Phe + Val + Met}}$$

in which ratios of 0.84–1.68 are assigned to non-membrane proteins, ratios of 0.41–0.77 are assigned to integral membrane proteins, and ratios of 1.02–1.72 are assigned to peripheral proteins.

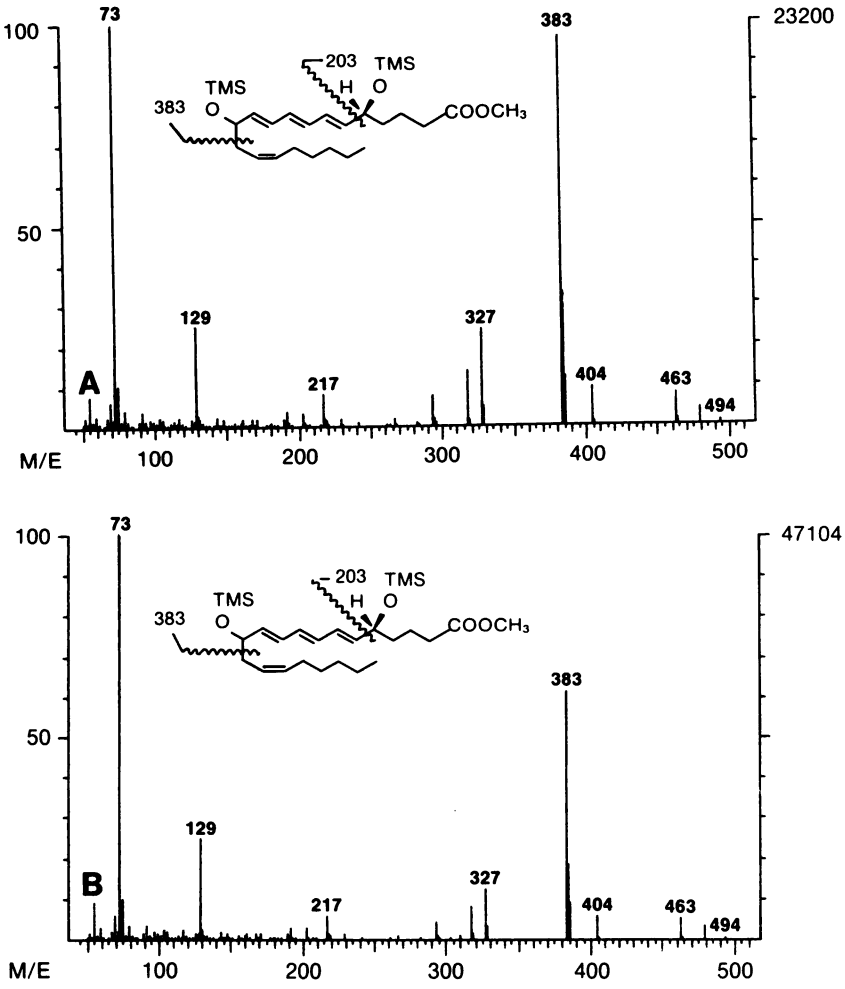

Fig. 7. Electron impact mass spectra of major diHETE products isolated from incubation of [^{14}C]arachidonate with purified 5-lipoxygenase. The incubation conditions are identical to those described in Fig. 5. The extraction and isolation of reaction products diHETE-1 (A) and diHETE-2 (B) for GC/MS are described in Experimental Procedures.

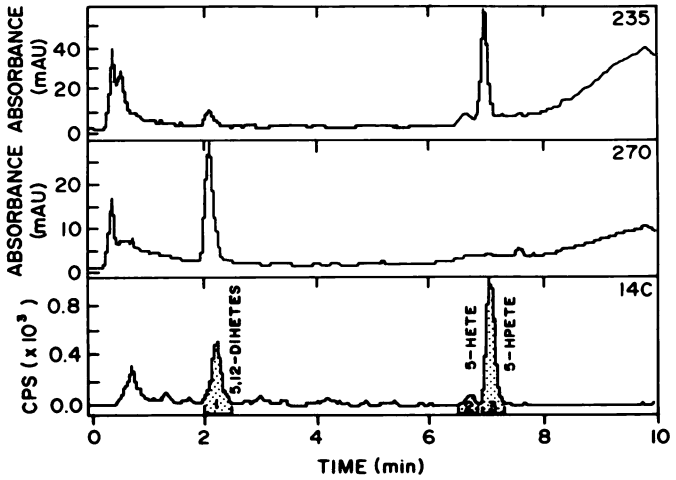

Fig. 8. RP-HPLC chromatograms of ^{14}C-reaction products produced from [^{14}C]5-HPETE by purified 5-lipoxygenase. The reaction conditions and analyses were identical to those described in Fig. 5 except that 5 μM [^{14}C]5-HPETE was incubated with the purified enzyme for 60 min. The chromatograms are a representative example of four separate experiments.

other enzymes involved in leukotriene biosynthesis including LTA_4 hydrolase (17) and glutathione *S*-transferase (18).

Identification of reaction products. Incubation of the purified 5-lipoxygenase with [^{14}C]arachidonate in the presence of 2 mM Ca^{2+} and ATP yielded the reaction products shown in Fig. 5. The 5-lipoxygenase rapidly converted [^{14}C]arachidonate to [^{14}C]5-HPETE and diHETEs. The 5-HPETE was identified by its retention time on RP-HPLC and scanning spectrophotometry (Fig. 6, *product A*). The diHETE peak was identified primarily as a mixture of two products. The reaction products were purified as described in Experimental Procedures and GC/MS was performed. The peaks diHETE I (Fig. 7A) and diHETE II (Fig. 7B) accounted for 33 and 47% of the recovered radioactivity in the diHETE region, respectively. The remainder of the radioactivity (10–15%) was not further separated and identified. Underivatized diHETE I had an HPLC elution volume identical to that of synthetic 12-epi-6-*trans*-LTB_4. Furthermore, the mass spectrum of diHETE I is consistent with a tetraunsaturated C_{20} fatty acid with hydroxyl groups at C_5 and C_{12}. Thus, diHETE I was assigned the structure of 5*S*,12*S*-dihydroxy-(*E*,*E*,*E*,*Z*)-6,8,10,14-eicosatetraenoic acid (12-epi-6-*trans*-LTB_4). Underivatized diHETE II had an HPLC elution volume identical to that of synthetic 6-*trans*-LTB_4. In addition, the mass spectrum of diHETE II is also consistent with a tetraunsaturated C_{20} fatty acid with hydroxyl groups at C_5 and C_{12}. Therefore, diHETE II was assigned the structure 5*S*,12*R*-dihydroxy-(*E*,*E*,*E*,*Z*)-6,8,10,14-eicosatetraenoic acid (6-*trans*-LTB_4). The scanning spectra of the products (Fig. 6, *product B*) were identical to those of either synthetic 6-*trans*-LTB_4 or synthetic 6-*trans*-12-epi-LTB_4.

The purified 5-lipoxygenase also converted [^{14}C]5-HPETE to [^{14}C]diHETEs (Fig. 8). The diHETE peak was identified

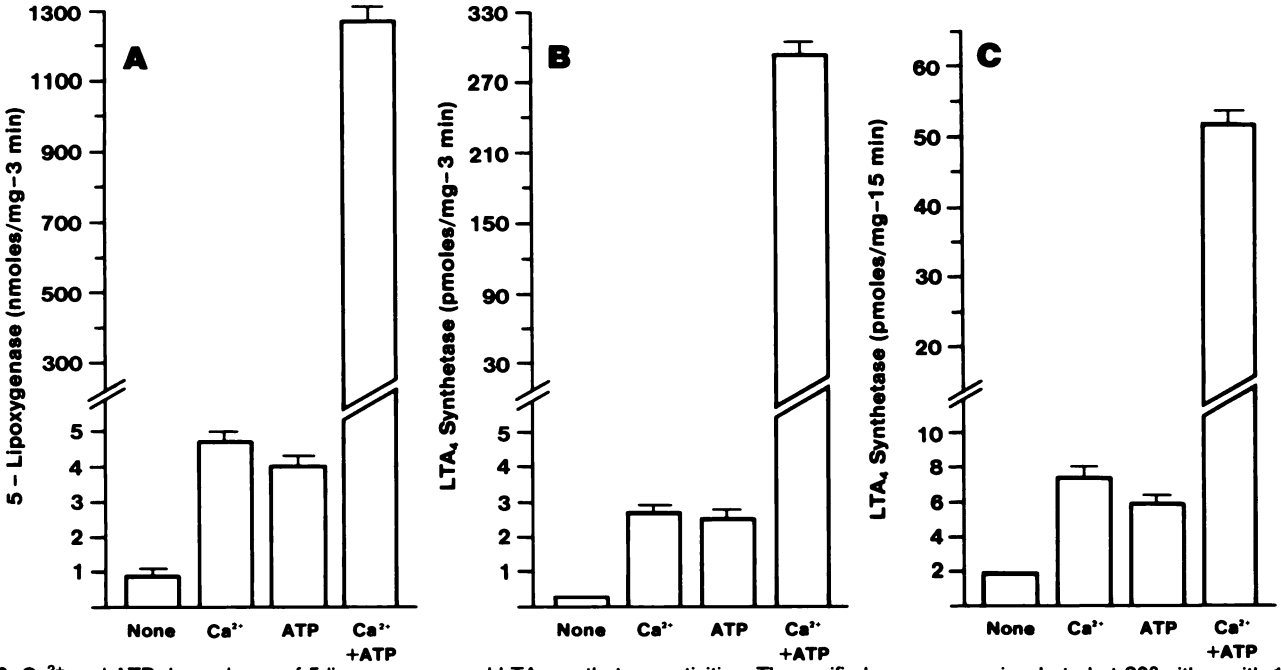

Fig. 9. Ca^{2+} and ATP dependence of 5-lipoxygenase and LTA_4 synthetase activities. The purified enzyme was incubated at 20° either with 10 μM [^{14}C]arachidonate for 3 min or 5 μM [^{14}C]5-HPETE for 15 min either in the presence or absence of 2 mM Ca^{2+} or 2 mM ATP. The reaction products were extracted and separated by RP-HPLC as described in Experimental Procedures. Activities were determined for 5-lipoxygenase (A), LTA_4 synthetase using [^{14}C]arachidonate as substrate (B), and LTA_4 synthetase using [^{14}C]5-HPETE as substrate (C). The data are expressed as the means ± standard errors of three separate experiments.

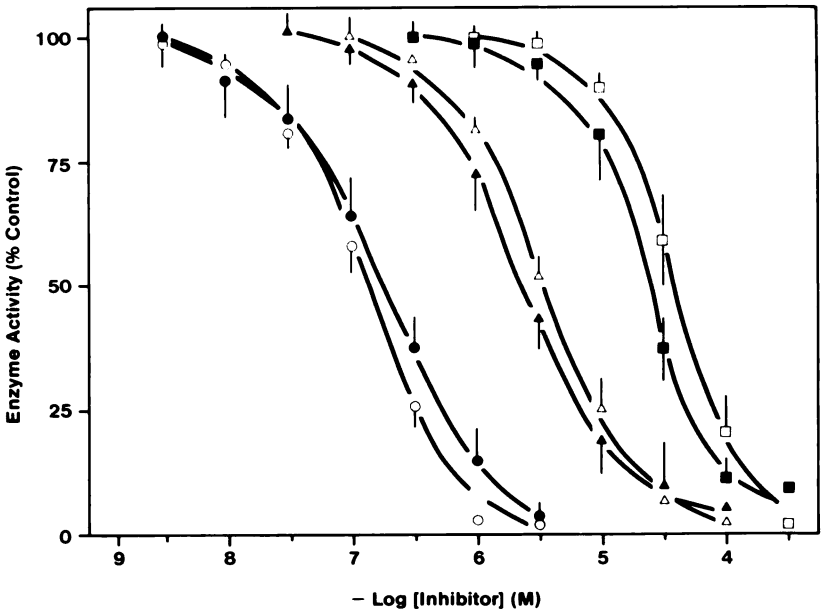

Fig. 10. Inhibition of 5-lipoxygenase and LTA_4 synthetase activities. Aliquots of the purified enzyme were incubated at 20° either with 10 μM [^{14}C]arachidonate for 3 min or 5 μM [^{14}C]5-HPETE for 15 min in the presence of 2 mM Ca^{2+} and 2 mM ATP with varying concentrations of either NDGA (○, ●), diphenyl disulfide, (△, ▲), or SK&F 86002 (□, ■). The inhibitors were preincubated in each instance with the enzyme for 1 min at 20° in the presence of 2 mM Ca^{2+} and 2 mM ATP. The reaction products were extracted and separated by RP-HPLC as described in Experimental Procedures. Activities were determined for 5-lipoxygenase (○, △, □) and LTA_4 synthetase (●, ▲, ■) using [^{14}C]5-HPETE as substrate. Control (100%) activity for 5-lipoxygenase and LTA_4 synthetase was 1051 ± 101 nmol/mg/min and 71 ± 9.1 pmol/mg/min, respectively. The data are expressed as the means ± standard errors of three separate experiments.

also as a mixture of two products. The two products accounted for 35 and 48% of the recovered radioactivity in the diHETE region, respectively. These products were identical to 6-*trans*-12-epi-LTB_4 and 6-*trans*-LTB_4 as determined by HPLC elution volumes, GC/MS, and scanning spectra (Fig. 6, *product C*). These data indicate that the homogeneous 5-lipoxygenase catalyzes two enzymatic reactions: the first by oxidation of arachidonate to produce 5(*S*)HPETE and the second by epoxidation of 5(*S*)HPETE to produce LTA_4. Thus, RBL-1 cell 5-lipoxygenase, as well as 5-lipoxygenase from human (12) and pig (8) leukocytes, functions as a single protein with dual enzyme activities.

Characteristics of 5-lipoxygenase and LTA_4 synthetase. Studies with high speed supernatants (16) and partially purified fractions (3, 4) of RBL-1 5-lipoxygenase have demonstrated activation with Ca^{2+} and ATP. Recent studies with highly purified 5-lipoxygenase from pig leukocytes (8) demonstrated an ATP-dependent activation of both 5-lipoxygenase and LTA_4 synthetase in the presence of 2 mM $CaCl_2$. Studies were designed, therefore, to determine the relative dependencies of the RBL-1 5-lipoxygenase and LTA_4 synthetase on Ca^{2+} and ATP.

As shown in Fig. 9A, the purified 5-lipoxygenase was stimulated slightly either by 2 mM Ca^{2+} (free Ca^{2+} of 20 μM in the

presence of 1 mM EDTA and in the absence of ATP) or 2 mM ATP (free Ca^{2+} concentration below 100 nM in the presence of 1 mM EDTA). In separate studies these concentrations of Ca^{2+} and ATP gave the maximal enzyme activation (data not shown, $N = 3$). However, in the presence of both Ca^{2+} and ATP, the 5-lipoxygenase activity was increased more than 300-fold when compared either to ATP alone or Ca^{2+} alone. The LTA_4 synthetase activity (Fig. 9B), measured using arachidonate as substrate, was also increased more than 100-fold in the presence of Ca^{2+} and ATP. By contrast, the LTA_4 synthetase activity (Fig. 9C), measured using 5-HPETE as substrate, was stimulated only 8-fold by Ca^{2+} and ATP. However, the latter LTA_4 synthetase activity was 50- to 60-fold less than the LTA_4 synthetase activity measured when 5-HPETE was endogenously synthesized from arachidonate by 5-lipoxygenase. This was also observed for highly purified pig leukocyte (6) LTA_4 synthetase. These data suggest that either arachidonate has a modulatory effect on LTA_4 synthetase or that the 5-HPETE endogenously synthesized is held in an energetically preferred conformation by the enzyme for conversion to LTA_4. The 5-HPETE free in solution may be in a less favorable conformation such that the catalytic rate of LTA_4 synthetase is reduced.

The effect of inhibitors on purified 5-lipoxygenase and LTA_4 synthetase activities was determined (Fig. 10). The compounds studied each produced a concentration-dependent inhibition of both activities. NDGA, a well characterized 5-lipoxygenase inhibitor with antioxidant properties, was the most potent with IC_{50} values for 5-lipoxygenase and LTA_4 synthetase of 130 and 178 nM, respectively. Diphenyl disulfide, a reversible inhibitor of 5-lipoxygenase from RBL-1 supernatants (19), inhibited 5-lipoxygenase and LTA_4 synthetase with IC_{50} values of 3.3 and 2.4 μM, respectively. SK&F 86002, a recently reported (20) orally active anti-inflammatory agent, inhibited 5-lipoxygenase with an IC_{50} of 35 μM and LTA_4 synthetase with an IC_{50} of 25 μM. Similar data for LTA_4 synthetase were obtained when arachidonate was used to synthesize 5-HPETE endogenously as substrate for the enzyme. These data indicate that the substrate-binding site for the two enzyme activities may be very similar structurally. However, whether arachidonate and 5-HPETE bind to the same or distinct catalytic sites cannot be inferred from the present data. Although arachidonate and 5-HPETE are structurally very similar, separate catalytic sites may be suggested on the basis of requirements for O_2, oxidation-reduction potentials for the substrates, and differences in catalytic activity of endogenously synthesized versus exogenously added 5-HPETE. Studies involving these concepts are currently in progress.

Discussion

The data in the present report demonstrate the rapid and reproducible purification of a single homogeneous protein from RBL-1 cells that contains both 5-lipoxygenase and LTA_4 synthetase activities. The amino acid composition is compatible with the molecular properties of the protein including hydrophobicity and anionic character and indicates that this protein is soluble and located in the cytosolic compartment of the RBL-1 cell. The N-terminal amino acid sequence of 30 amino acids shows that the enzyme is a homogeneous protein chain. The hydrophobicity ratio of 0.5 for the first 30 amino acids suggests that, although the protein as a macromolecule is cytosolic in nature, the N-terminus of the protein may be membrane associated. The 5-lipoxygenase/LTA_4 synthetase from RBL-1 cells has characteristics similar to those of highly purified preparations of enzyme for human (7) and pig (8) leukocytes, including molecular weight (72,000–75,000), Ca^{2+} and ATP dependence, low catalytic activity of LTA_4 synthetase with exogenous 5-HPETE versus endogenously synthesized 5-HPETE, and inhibition by various lipoxygenase inhibitors.

The present purification scheme is a substantial improvement over previous reports (6–9, 12) in that the enzyme was reproducibly purified to homogeneity in a stable form in good yield with high specific activity. Previous reports with highly purified enzyme either had low yields (less than 1%) or did not quantitate specific activity because of enzyme instability. The 5-lipoxygenase purified from RBL-2H3 cells (6) lacked LTA_4 synthetase activity, was not Ca^{2+} dependent, and had comparably lower specific activity. The higher specific activity found in this study suggests that the RBL-1 5-lipoxygenase/LTA_4 synthetase may only require Ca^{2+} and ATP as cofactors and the multiple regulatory factors reported for the human leukocyte enzyme (7, 9, 12) may be of no relevance in this system.

The stability of 5-lipoxygenase/LTA_4 synthetase was optimized in the presence of 5–15% ethylene glycol, 1 mM EDTA, and 20–100 μg/ml sonicated phosphatidylcholine. As reported originally by Goetze *et al.* (6), sonicated phosphatidylcholine micelles were used throughout the purification as a stabilizing agent. The phosphatidylcholine was not required for activity as reported for microsomal membrane factor(s) which stimulate human leukocyte 5-lipoxygenase (7, 9, 12). Phosphatidylcholine has been reported to be oxygenated by 15-lipoxygenase but not 5-lipoxygenase (21). Thus, the phosphatidylcholine micelles may preserve 5-lipoxygenase/LTA_4 synthetase activity by interacting with the substrate-binding site in a manner that alters the conformation of the enzyme to a more stable form. Alternately, the lipid micelles may associate with hydrophobic regions of the enzyme that are sensitive to either proteolysis or denaturation. The metal chelating agent, EDTA, provides stability, probably through inhibition of metal-dependent proteolysis. In addition, the 5-lipoxygenase/LTA_4 synthetase, either in crude or purified form, was observed to autoinactivate in a time-dependent manner in the presence of CA^{2+} (data not shown, $N = 5$). By chelating free Ca^{2+}, EDTA may prevent the inactivation process.

The availability of homogeneous 5-lipoxygenase/LTA_4 synthetase provides the basis for the development of molecular biological tools for further understanding of the role of this complex and important enzyme in the regulation of leukotriene biosynthesis. To this end, polyclonal antibodies to RBL-1 5-lipoxygenase/LTA_4 synthetase and oligonucleotides coding for the N-terminal amino acid sequence have been prepared (data not shown). At present, cellular 5-lipoxygenase may only be studied at the level of enzymatic products such as 5-HETE, LTB_4, and the peptidoleukotrienes. Recent studies with human leukocytes (22) and human eosinophils (23) have indicated that cellular products of 5-lipoxygenase/LTA_4 synthetase increase in disease states such as asthma. In addition, HL-60 leukemia cells produce substantially larger amounts of 5-lipoxygenase/LTA_4 synthetase products when differentiated with *N,N*-dimethylformamide (24). These studies suggest that 5-lipoxygenase/LTA_4 synthetase may be regulated at the molecular level as a consequence of pathophysiological stimuli. Thus, molecular probes may be utilized to study the dynamic regulation of the

enzyme and provide more direct information regarding the role and importance of 5-lipoxygenase/LTA_4 synthetase in inflammatory disease states.

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