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Purification to homogeneity and the N-terminal sequence of human leukotriene C_4 synthase: A homodimeric glutathione *S*-transferase composed of 18-kDa subunits

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ABSTRACT **Human leukotriene C_4 (LTC_4) synthase was purified >25,000-fold to homogeneity from the monocytic leukemia cell line THP-1. Beginning with taurocholate-solubilized microsomal membranes, LTC_4 synthase was chromatographically resolved by (*i*) anion exchange, (*ii*) affinity chromatography (through a resin of biotinylated LTC_2 immobilized on streptavidin-agarose), and then (*iii*) gel filtration. The final preparation contained only an 18-kDa polypeptide. The molecular mass of the pure polypeptide was consistent with an 18-kDa polypeptide from THP-1 cell membranes that was specifically photolabeled by an LTC_4 photoaffinity probe, ^{125}I-labeled azido-LTC_4. On calibrated gel-filtration columns, purified LTC_4 synthase activity eluted at a volume corresponding to 39.2 ± 3.3 kDa (n = 12). The sequence of the N-terminal 35 amino acids was determined and found to be a unique sequence composed predominantly of hydrophobic amino acids and containing a consensus sequence for protein kinase C phosphorylation. We therefore conclude that human LTC_4 synthase is a glutathione *S*-transferase composed of an 18-kDa polypeptide that is enzymatically active as a homodimer and may be phosphoregulated *in vivo*.**

Leukotrienes (LTs) are potent lipid-derived mediators that are released in response to a variety of immunologic and inflammatory stimuli (1, 2). The enzyme LTC_4 synthase catalyzes the first committed step in the biosynthesis of the cysteinyl LTs (LTC_4, LTD_4, and LTE_4) that collectively make up the slow-reacting substance of anaphylaxis and appear to play a pivotal role in the pathogenesis of human bronchial asthma (3). LTC_4 synthase is a membrane-bound glutathione *S*-transferase activity that is distinct from α, μ, π, θ, and microsomal glutathione *S*-transferases and appears to be exclusively committed to the biosynthesis of LTC_4 (4). Of the known human glutathione *S*-transferases, it is the only one that has not yet been purified to homogeneity or cloned, due predominantly to the extreme lability of LTC_4 synthase in partially purified forms and the lack of a suitably abundant source of the enzyme.

Recently, we have described the induction of high levels of LTC_4 synthase activity in the human promonocytic leukemia cell line U937 after differentiation into monocyte/macrophage-like cells by growth in the presence of dimethyl sulfoxide (Me_2SO) (4). From the microsomal membranes of these cells, LTC_4 synthase was purified >10,000-fold and an 18-kDa membrane polypeptide was identified by photoaffinity labeling as a strong candidate for being LTC_4 synthase or a subunit of the enzyme (5). In this report, we describe the purification to homogeneity of human LTC_4 synthase from the monocytic leukemia cell line THP-1 and show that like other known glutathione *S*-transferases, which are all low molecular mass dimeric or trimeric enzymes, human LTC_4 synthase is composed of a single 18-kDa polypeptide that is functionally active as a homodimer.

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MATERIALS AND METHODS

Cell Growth and Subcellular Fractionation. U937 and HL-60 cells were grown and differentiated as described (4). Cells from the human monocytic leukemia cell line THP-1 (American Type Culture Collection TIB 202; ref. 6) were propagated in culture similarly except the medium was RPMI 1640 (supplemented with 0.2% $NaHCO_3$, 0.03% L-glutamine, and 50 μM 2-mercaptoethanol) containing 10% (vol/vol) fetal bovine serum (Sigma Hybri-Max, not heat-inactivated), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were harvested after 4–7 days in culture by continuous-flow centrifugation and ruptured by nitrogen cavitation (30 min at 800 psi; 1 psi = 6.9 kPa), and the 100,000 × g microsomal membrane fraction was isolated as described (5).

Preparation of Membranes from Human Lung. Postmortem human lung samples were obtained from the International Institute for the Advancement of Medicine (Essington, PA). The 100,000 × g membrane fraction was isolated from homogenized parenchyma essentially as described (7) except that the homogenization and resuspension buffer was 0.25 M sucrose/10 mM Mops·KOH, pH 7.4/2 mM EDTA containing 2 mM phenylmethylsulfonyl fluoride (added from a fresh 200 mM stock in ethanol).

Photoaffinity Labeling of THP-1 Cell Membranes. The preparation of ^{125}I-labeled azido-LTC_4, a radioiodinated photolabile derivative of LTC_4, and photoaffinity labeling of THP-1 cell microsomal membranes were essentially as described for the photolabeling of U937 cell membranes (4).

Purification of Human LTC_4 Synthase from THP-1 Cells. LTC_4 synthase activity was measured essentially as described (5) by the formation of LTC_4 in incubation mixtures containing reduced glutathione and the free acid of LTA_4. Unless otherwise indicated, all purification procedures were performed either at 4°C or on ice.

Solubilization of LTC_4 synthase with taurocholate. Membrane-bound LTC_4 synthase activity was solubilized with 2% (wt/vol) taurocholate (Calbiochem) as described (5).

Chromatographic step 1: Anion-exchange chromatography. The taurocholate extract of THP-1-cell microsomal membranes (50 ml, containing 500–800 mg of protein) was

Abbreviations: LT, leukotriene; Me_2SO, dimethyl sulfoxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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injected onto a HiLoad Q Sepharose HP 26/10 anion-exchange column (Pharmacia, 2.6 × 10 cm) that had been equilibrated in buffer A {20 mM Tris·HCl, pH 7.4/1 mM EDTA/2 mM reduced glutathione/1 mM dithiothreitol/0.1% taurocholate/0.5% *n*-octyl glucoside (Boehringer Mannheim)/0.5% 3-[(3-cholamidopropyl)dimethylammonia]-1-propane sulfonate (CHAPS, Calbiochem)} at a flow rate of 10 ml/min. After washing the column with 300 ml of buffer A, bound proteins were eluted with a linear gradient of NaCl (0–1.0 M, 1200-ml gradient volume) in buffer A.

Chromatographic step 2: LTC_2 affinity chromatography. The preparation of LTC_2-X-biotin–streptavidin-agarose affinity resins has been described (5). LTC_2, a stable analogue of the LTC_4 synthase enzymatic product LTC_4, was synthesized for use as an affinity ligand essentially as described (8). Biotin was linked to the α-amino group of the γ-glutamate component of the LTC_2 glutathione moiety by incubation with an *N*-hydroxysuccinimide ester of biotin having a spacer arm length of 2.24 nm [NHS-X-biotin; succinimidyl 6-(biotinamido)hexanoic acid; Molecular Probes] to form LTC_2-X-biotin. LTC_2-X-biotin was then immobilized on streptavidin-agarose at a ligand concentration of 40 μM. The resulting LTC_2-X-biotin–streptavidin-agarose affinity resin was packed into a Pharmacia HR 10/2 FPLC column (10 mm diameter × 4.5 mm bed height) and preequilibrated at 1.0 ml/min in running buffer B [20 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM dithiothreitol/0.1% taurocholate/0.5% *n*-octyl glucoside/0.5% CHAPS (similar to running buffer A except without reduced glutathione)]. The active fractions from the anion-exchange step were pooled (typically 20 ml in total) and diluted 1:5 into running buffer B to reduce NaCl and glutathione concentrations, both of which inhibited LTC_4 synthase binding to the affinity resin. The preparation was then injected onto the LTC_2 affinity column at a flow rate of 0.2 ml/min. After sample application, the flow rate was returned to 1.0 ml/min and the column was washed with 10 ml of running buffer B. LTC_4 synthase was eluted with a linear NaCl/reduced glutathione cogradient (0–1.0 M NaCl and 0–4 mM reduced glutathione; 15-ml gradient in buffer B).

Chromatographic step 3: Gel-permeation chromatography. The active fractions from two LTC_2 affinity column runs were combined (typically 6 ml in total), then concentrated on a YM10 ultrafiltration membrane (Amicon) to ≤0.25 ml, and subsequently injected onto a Superdex 75 HR 10/30 column (Pharmacia, 1 × 30 cm) that had been equilibrated in buffer C [20 mM Tris·HCl, pH 7.4/1 mM EDTA/2 mM reduced glutathione/1 mM dithiothreitol/0.1% taurocholate/0.5% *n*-octyl glucoside/0.5% CHAPS/0.25 M NaCl (similar to buffer A except also containing NaCl)]. LTC_4 synthase activity was eluted isocratically at a flow rate of 0.25 ml/min. The Superdex 75 column was calibrated by performing an identical chromatographic separation of LTC_4 synthase coinjected with a mixture of molecular mass standards (each at 0.4 mg). Fractions were collected throughout the chromatogram and the molecular mass standards were positively identified on silver-stained SDS/polyacrylamide gels. Column calibration was performed by constructing a standard curve of the known molecular mass of the standards vs. their respective partition coefficients, K_{av} [where $K_{av} = (V_e - V_o)/(V_t - V_o)$; V_e is the elution volume, V_o is the void volume of the column, and V_t is the total bed volume of the column]. The resulting sigmoidal plot of logarithmic molecular mass vs. K_{av} was used to calculate the native molecular mass of LTC_4 synthase based on the elution volume of enzymatic activity. Calibration and chromatographic separation on Superose 12 HR 10/30 columns (Pharmacia, 1 × 30 cm) were performed in an identical manner.

N-Terminal Sequence Determination. Fractions containing the pure 18-kDa LTC_4 synthase polypeptide (≈100 pmol) were pooled and incubated at room temperature for 48 h with end-over-end mixing in a tube that also contained a 3 × 5 mm sliver of a poly(vinylidene difluoride) membrane. The sliver was washed several times with water then used directly for sequencing. Conventional Edman degradation microsequencing was performed on a modified Applied Biosystems model 477A gas-phase sequencer equipped with a continuous-flow reactor (9).

Miscellaneous Methods. Human monocytes were isolated from the blood of healthy human volunteers by using discontinuous Histopaque gradients as described (4). Glutathione *S*-transferase activity was measured spectrophotometrically (10). Microsomal glutathione *S*-transferase activity was measured in the presence of Triton X-100 after *N*-ethylmaleimide pretreatment (11, 12). SDS/PAGE was performed using standard methods (13) and protein bands in gels were visualized by silver staining (14). Silver-staining artifact bands (15) were identified in blank lanes containing SDS sample buffer only. Protein was determined as described (16) using bovine γ-globulin as standard. In highly purified LTC_4 synthase preparations where protein determination was not possible by this method, protein was estimated by laser densitometry of samples on silver-stained SDS/polyacrylamide gels. Where indicated, LTC_4 synthase activity is expressed as the mean ± SD.

RESULTS

THP-1 Cells Are an Abundant Source of LTC_4 Synthase Activity. Human lung parenchyma membranes were found to contain a substantial amount of LTC_4 synthase activity (316.5 ± 71.6 pmol of LTC_4 formed per min per mg of protein, n = 4; Table 1). We have observed (4) that the specific activity of LTC_4 synthase in the human promonocytic leukemia cell line U937 (39.9 ± 16.7 pmol of LTC_4 formed per min per mg; n = 8) was elevated 10-fold (to 399.0 ± 84.1 pmol of LTC_4 formed per min per mg; n = 47) concomitantly with Me_2SO-induced differentiation into monocyte/macrophage-like cells and was thus comparable to that found in human lung. We therefore tested for LTC_4 synthase activity in related cell lines of monocytic lineage and found endogenously high levels of LTC_4 synthase in the monocytic leukemia cell line THP-1 (302.0 ± 62.0 pmol of LTC_4 formed per min per mg; n = 9) without further differentiation.

Purification of Human LTC_4 Synthase. As with Me_2SO-differentiated U937 cells (4, 5), LTC_4 synthase activity in THP-1 cells was predominantly localized in the 100,000 × g microsomal membrane fraction and could be solubilized with high recovery (>90%) by the anionic detergent taurocholate

Table 1. LTC_4 synthase activity in cells of human origin

Source	LTC_4 synthase specific activity, pmol of LTC_4 formed per min per mg
Human cell line	
U937	39.9 ± 16.7 (8)*
dU937/Me_2SO	399.0 ± 84.1 (47)*
dU937/PMA	45.9 ± 8.4 (5)
HL-60	6.8 ± 2.3 (4)
dHL-60/Me_2SO	30.7 ± 12.7 (3)
dHL-60/PMA	50.5 ± 17.3 (3)
THP-1	302.0 ± 62.0 (9)
Human tissue	
Monocytes	21.5 ± 4.8 (5)*
Lung membranes	316.5 ± 71.6 (4)

Specific activity of LTC_4 synthase was determined in human cell lines, cells from peripheral human blood, or membranes from human lung parenchyma. For human cell lines (U937, HL-60, and THP-1), the prefix d indicates they were differentiated in culture in the presence of Me_2SO or phorbol 12-myristate 13-acetate (PMA) as indicated. Data are the mean ± SD; numbers in parentheses are n. *Data are derived from ref. 14.

(data not shown). A mixture of detergents, 0.1% taurocholate/0.5% *n*-octyl glucoside/0.5% CHAPS, was found to work well in all chromatographic steps and enabled the purification of LTC_4 synthase from THP-1 cell membranes to homogeneity as described below.

In the first chromatographic step (anion exchange), taurocholate extract from THP-1 cell microsomal membranes (50 ml, containing 500–800 mg of protein) was applied to a quaternary aminoethyl anion-exchange column. The column was developed with a linear gradient of NaCl and LTC_4 synthase activity was eluted at ≈0.2 M NaCl (Fig. 1). The specific activity of LTC_4 synthase was increased 25- to 35-fold over the taurocholate extract used as starting material and the yield in this step was generally 40–60%.

In a previous report (5), we described the development of an affinity column, LTC_2-X-biotin–streptavidin-agarose, to which LTC_4 synthase could be bound and eluted in an active form. The affinity ligand LTC_2 (a stable analog of the product of the LTC_4 synthase-catalyzed reaction) was first biotinylated via the primary amino group on the γ-glutamate residue of the LTC_2 glutathione moiety and then the biotinylated LTC_2 was immobilized on streptavidin-agarose at 40 μM. The column containing the LTC_2-X-biotin–streptavidin-agarose was equilibrated in a chromatography buffer that was identical to that used for anion-exchange except that glutathione was omitted since it competed with the LTC_2 affinity ligand for LTC_4 synthase binding. The active fractions from anion-exchange chromatography were pooled, diluted 1:5 with chromatography buffer (to reduce the concentration of NaCl and glutathione, both of which prevented LTC_4 synthase binding to the affinity column), and passed on to the affinity column at a low flow rate (Fig. 2). The vast majority of the total protein went through the affinity column, whereas all of the LTC_4 synthase activity was bound. The column was developed with a NaCl/reduced glutathione cogradient and LTC_4 synthase activity was eluted at ≈0.3 M NaCl/1.2 mM reduced glutathione. LTC_4 synthase activity was enriched 50- to 60-fold by this step with a yield of 5–15%.

The final chromatographic step in the purification of human LTC_4 synthase from THP-1 cell membranes was gel permeation chromatography through a Pharmacia Superdex 75 column (exclusion limit = M_r 100,000; selectivity range = M_r 3000–70,000). The active fractions from LTC_2 affinity chromatography were pooled, concentrated to ≤250 μl by ultrafiltration using a YM10 membrane (Amicon), and then injected onto the column (Fig. 3). LTC_4 synthase specific activity was enriched a further 3- to 5-fold over the active fraction from the LTC_2 affinity column, used as starting material, with a recovery of 20–30%.

Properties of Purified Human LTC_4 Synthase. A representative purification series is summarized in Table 2. Beginning with 2×10^{11} THP-1 cells, LTC_4 synthase was purified >27,000-fold over intact cells. The yield was <1% due in part to the intentional use of only the very center fractions (generally accounting for less than or equal to two-thirds) of the activity peaks. The final preparation consisted of a single polypeptide of ≈18 kDa (Fig. 4). It was judged to be homogeneous based on the presence of a single protein band on silver-stained SDS-denaturing gels (Fig. 4*b*) and on isoelectric focusing gels (data not shown) and by the presence of single phenylthiohydantoin amino acid products after each cycle of sequential Edman degradation (see below). Approximately 3.5 μg of the purified 18-kDa polypeptide (≈200 pmol) was generated by this procedure from 2×10^{11} THP-1 cells. (This was reproducible in other preparations within 25%.) The V_{max} of the final preparation was 4.1 μmol of LTC_4 formed per min per mg (Table 2) and the K_m for LTA_4 was 9.9 μM whereas the K_m for reduced glutathione was 1.7 mM (data not shown). This was in close agreement with the K_m values for LTC_4 synthase in Me_2SO-differentiated U937 cells and in human blood monocytes (5). Human LTC_4 synthase did not have detectable glutathione *S*-transferase activity in the presence of reduced glutathione plus 1-chloro-2,4-

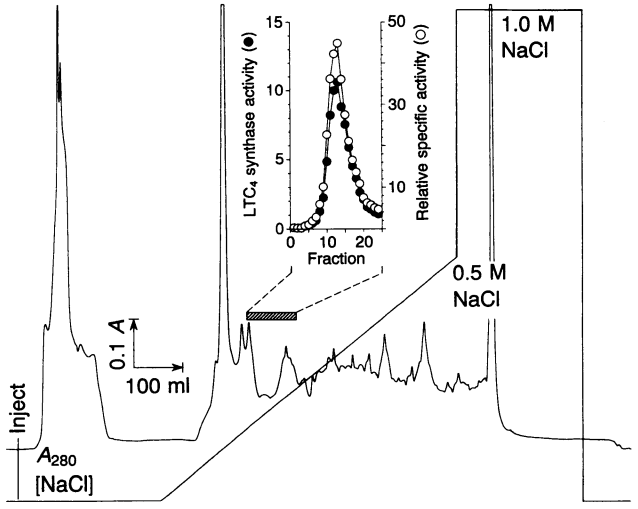

FIG. 1. Anion-exchange chromatography. A taurocholate extract (50 ml) of the 100,000 × *g* membrane fraction from THP-1 cells was applied to a HiLoad 26/10 Q Sepharose anion-exchange column that had been equilibrated in buffer A. The column was developed with a linear gradient of NaCl in buffer A and was monitored by on-line measurement of A_{280} as indicated. Fractions (4 ml) were collected and subsequently assayed for LTC_4 synthase activity (nmol of LTC_4 formed per min per ml) in standard incubation mixtures. LTC_4 synthase activity (●) was eluted from the column at ≈0.2 M NaCl in the region indicated by the hatched bar (to which *Inset* corresponds). The relative specific activity of LTC_4 synthase in each fraction (○) was calculated with respect to the LTC_4 synthase specific activity of the taurocholate extract used as starting material, which was set at 1.

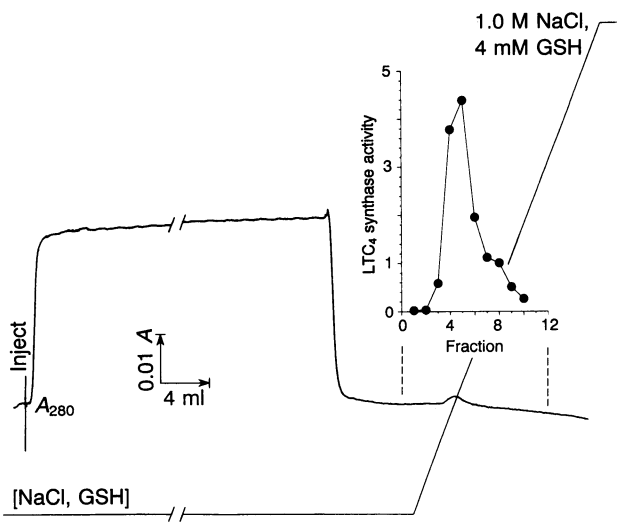

FIG. 2. LTC_2 affinity chromatography. Active fractions from anion-exchange chromatography were pooled, diluted 1:5 in buffer B, and applied to an LTC_2 affinity column (constructed by immobilizing biotinylated LTC_2 on streptavidin-agarose) that had been equilibrated in buffer B. The column was developed with a linear NaCl/reduced glutathione cogradient in buffer B and monitored by on-line measurement of A_{280} as indicated. Note the gap indicated in the chromatogram during loading corresponds to 20 ml. Fractions (1 ml) were collected and subsequently assayed for LTC_4 synthase activity (nmol of LTC_4 formed per min per ml) in standard incubation mixtures. LTC_4 synthase activity (*Inset*) was eluted at 0.3 M NaCl/1.2 mM reduced glutathione.

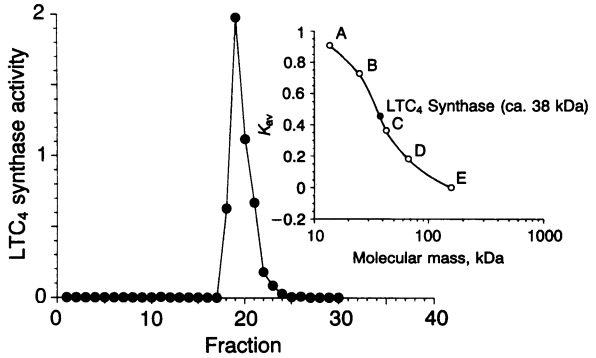

FIG. 3. Gel-filtration chromatography. The active fractions from LTC_2 affinity chromatography (two runs) were pooled, concentrated by ultrafiltration, and then applied to a Superdex 75 gel-filtration column that had been equilibrated in buffer C. Proteins were eluted isocratically with buffer C. Fractions (0.5 ml) were collected and subsequently assayed for LTC_4 synthase activity (●; nmol of LTC_4 formed per min per ml). (*Inset*) The Superdex 75 column was calibrated and the native molecular mass of LTC_4 synthase was determined by performing an identical chromatographic separation on LTC_4 synthase coinjected with a mixture of standards. The calibration curve was constructed by plotting the logarithm of the known molecular mass of the standards (○) vs. their respective partition coefficient (K_{av}) on the column. LTC_4 synthase activity (●) was eluted at a volume corresponding to 38 kDa. Standards: A, RNase A (13.7 kDa); B, chymotrypsinogen (25 kDa); C, ovalbumin (43 kDa); D, albumin (67 kDa); E, aldolase (158 kDa).

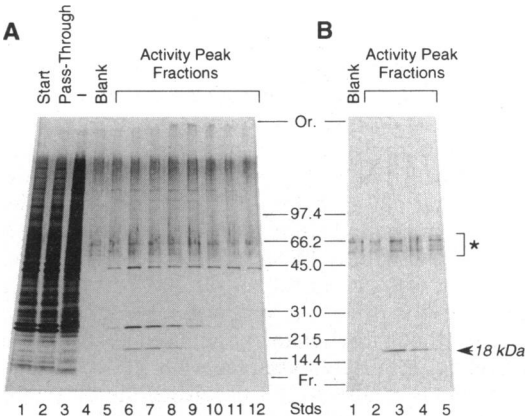

FIG. 4. Polypeptide constituents of chromatography fractions. Various fractions from LTC_2 affinity chromatography (*A*) or Superdex 75 gel filtration (*B*) were dissociated in SDS-containing sample buffer and then resolved by SDS/PAGE (8–16% polyacrylamide gradient gels). Protein bands were visualized by silver staining. Each gel included a sample-buffer-only blank (*A*, lane 4; *B*, lane 1) to identify artifact bands resulting from silver staining, which are indicated by an asterisk. The migration of molecular mass standards (in kDa), the origin (Or.) and front (Fr.) are indicated in lane Stds. (*A*) LTC_2 affinity chromatography. Equal volumes of the anion-exchange pool used as starting material for LTC_2 affinity chromatography (lane 1), the column pass-through (lane 2), and the fractions from the elution gradient in the region where LTC_4 synthase activity was detected (lanes 5–12) were resolved. LTC_4 synthase activity peaked in the fraction shown in lane 7. (*B*) Gel filtration. Equal volumes of the fractions in the region where LTC_4 synthase activity was eluted from the column were resolved. LTC_4 synthase activity peaked in the fraction shown in lane 3.

dinitrobenzene, 4-nitrobenzyl chloride, or 1,2-epoxy-3-(4-nitrophenoxy)propane (data not shown).

LTC_4 Synthase Is a Homodimer Composed of 18-kDa Subunits. As described above and in Fig. 4*B*, the >25,000-fold purified preparation of LTC_4 synthase was composed of a single 18-kDa polypeptide. This polypeptide corresponds to an 18-kDa polypeptide from Me_2SO-differentiated U937 cell membranes that was specifically photolabeled by ^{125}I-labeled azido-LTC_4, a radioiodinated photoreactive LTC_4 photoaffinity probe (4, 5). An apparently identical 18-kDa polypeptide was the only protein specifically labeled by ^{125}I-labeled azido-LTC_4 in THP-1 cell membranes (A.A., R.J.Z., A.W.F.-H., and D.W.N., unpublished data). The ability of various LTs to competitively inhibit LTC_4 synthase activity in THP-1 cell microsomal membranes ($LTC_2 \geq LTC_4 > LTD_4 > LTE_4 > LTB_4$; A.A. *et al.*, unpublished data) corresponded exactly with their ability to compete for photolabeling of the 18-kDa polypeptide in THP-1 cell membranes (Fig. 5), lending further support to the identification of the 18-kDa polypeptide as LTC_4 synthase. On calibrated gel-filtration columns, however, purified LTC_4 synthase activity was eluted at a volume corresponding to 39.2 ± 3.3 kDa ($n = 12$), twice the molecular mass of the 18-kDa polypeptide constituent of the active fractions (see Fig. 3 *Inset*). This was the case for two gel-filtration media where the native molecular mass was determined to be 37.5 ± 3.1 kDa (Pharmacia Superose 12; $n = 5$) and 40.4 ± 3.2 kDa (Pharmacia Superdex 75; $n = 7$). Although it is possible that the native molecular mass for LTC_4 synthase was overestimated due to artifacts sometimes associated with the gel-filtration chromatography of detergent-solubilized membrane proteins, this does not appear to be the case since in some preparations of LTC_4 synthase where activity was lost after LTC_2 affinity chromatography, the 18-kDa polypeptide was eluted later at a volume corresponding to 15–20 kDa. Enzymatically active LTC_4 synthase, therefore, appears to be a homodimer composed of two identical 18-kDa subunits.

N-Terminal Sequence of Human LTC_4 Synthase. The pure 18-kDa LTC_4 synthase polypeptide (≈100 pmol, purified from 2×10^{11} THP-1 cells) was adsorbed onto a sliver of poly(vinylidene difluoride) membrane and the N-terminal sequence of the first 35 amino acids, representing an estimated 20% of the 18-kDa polypeptide, was determined by automated Edman degradation (Fig. 6). The sequence was unique and was not homologous to any known sequences contained in available databases (as of January 27, 1993;

Table 2. Summary of a representative LTC_4 synthase purification from THP-1 cells

Fraction	Volume, ml	Activity, nmol per min per ml	Total activity, nmol/min	Recovery, %	Protein, mg/ml	Total protein, mg	Specific activity, nmol per min per mg	Relative specific activity
Harvested THP-1 cells	2000	4.36	8728	100	28.9	57,800	0.15	1.0
100,000 × *g* membranes	150	16.1	2418	27.7	40.0	6,000	0.40	2.7
Taurocholate extract	270	8.26	2230	25.5	13.2	3,554	0.63	4.2
HiLoad Q anion exchange	100	9.83	982.5	11.3	0.49	49.0	20.6	137
LTC_2 affinity	30	2.50	74.76	0.86	0.0023	0.069	1084	7,178
Superdex 75 gel filtration	11	1.38	15.16	0.17	0.00033	0.0037	4135	27,384

LTC_4 synthase was purified from 2×10^{11} THP-1 cells. The yield (percent recovery) was calculated from the total activity in cells, which was set at 100%. The relative specific activity was calculated with respect to the LTC_4 synthase specific activity in cells, which was set at 1.0.

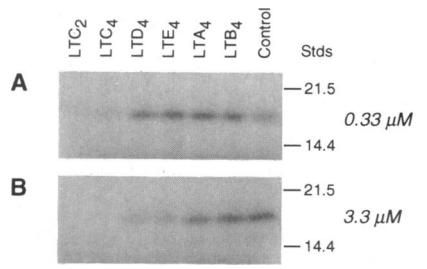

FIG. 5. Photoaffinity labeling of an 18-kDa polypeptide in the THP-1-cell 100,000 × *g* membrane fraction. THP-1-cell microsomal membranes (0.3 mg of protein) were incubated with 20 pM ^{125}I-labeled azido-LTC_4 in the absence (Control) or presence of 0.33 μM (*A*) or 3.3 μM (*B*) competing LTC_2, LTC_4, LTD_4, LTE_4, LTA_4, or LTB_4, as indicated, for 30 min at 25°C. After photolysis, labeled proteins were resolved by SDS/PAGE and visualized by autoradiography. The specifically labeled 18-kDa polypeptide is shown (molecular mass standards in kDa are indicated). Lanes were organized by the rank order of potency of the indicated LTs to competitively inhibit LTC_4 synthase activity in standard incubation mixtures (which was $LTC_2 \geq LTC_4 > LTD_4 > LTE_4 > LTB_4$; LTA_4, cosubstrate for LTC_4 synthase, could not be tested for inhibition).

nonredundant protein sequence databases at the National Center for Biotechnology Information using the BLAST network service). The protein kinase C consensus phosphorylation sequence Ser-Ala-Arg (17) was present at amino acid positions 28–30. A methionine residue was present in position 1, indicating that LTC_4 synthase is probably not synthesized as a larger precursor polypeptide.

DISCUSSION

LTC_4 synthase is a key enzyme in the synthesis of biologically active LTs. Since it catalyzes the first committed enzymatic step in the formation of the cysteinyl LTs, which make up the slow-reacting substance of anaphylaxis, its activity may substantially affect the profile of these inflammatory mediators in pathological situations.

LTC_4 synthase catalyzes a glutathione *S*-transferase reaction in which reduced glutathione is conjugated to the precursor LTA_4 to form LTC_4. As such, LTC_4 synthase may be a member of the glutathione *S*-transferase multigene family of enzymes that so far, with the exception of LTC_4 synthase, are involved in cellular detoxification events (for review, see refs. 18 and 19). Except for LTC_4 synthase, these glutathione *S*-transferases have been purified and in most cases cloned. We have demonstrated (4) that human LTC_4 synthase is a membrane-bound enzymatic activity that is completely distinct from α, μ, π, θ, and microsomal glutathione *S*-transferases. A partial purification of the enzyme (>10,000-fold) from the microsomal membranes of Me_2SO-differentiated U937 cells was enabled by the finding that enriched LTC_4 synthase preparations (>500-fold) were dependent on the presence, in LTC_4 synthase incubation mixtures, of Mg^{2+} and phosphatidylcholine for activity (5). In the current study,

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 M   K   D   E   V   A   L   L   A   A   V   T   L   L   G
Met-Lys-Asp-Glu-Val-Ala-Leu-Leu-Ala-Ala-Val-Thr-Leu-Leu-Gly-  15

 V   L   L   Q   A   G   F   S   L   Q   V   I   S   A   R
Val-Leu-Leu-Gln-Ala-Gly-Phe-Ser-Leu-Gln-Val-Iso-Ser-Ala-Arg-  30

  I   A   F   R   V
[Iso]-Ala-Phe-Arg-Val-
[Arg]
```

FIG. 6. N-terminal sequence of human LTC_4 synthase. Purified human LTC_4 synthase was sequenced by automated Edman degradation. Beginning with the N-terminal methionine, amino acids are listed by their three-letter abbreviations below their single-letter abbreviations. The residue at position 31 could not be distinguished between isoleucine and arginine. A potential protein kinase C phosphorylation site at positions 28–30 is underlined.

we have purified LTC_4 synthase >25,000-fold to homogeneity from the human monocytic leukemia cell line THP-1 and found that LTC_4 synthase has some similarities with other members of the glutathione *S*-transferase family of enzymes. In particular, LTC_4 synthase is enzymatically active as a homodimer of two 18-kDa subunits, a common feature of other glutathione *S*-transferases that are multimeric enzymes composed of low molecular mass subunits (11, 18, 19).

That the purified 18-kDa polypeptide is LTC_4 synthase was further supported by photoaffinity labeling studies using ^{125}I-labeled azido-LTC_4. In crude membrane preparations from both Me_2SO-differentiated U937 cells (4, 5) and THP-1 cells (A.A. *et al.*, unpublished data), ^{125}I-labeled azido-LTC_4 specifically labeled an 18-kDa polypeptide that was elevated during Me_2SO-induced differentiation of U937 cells concomitantly with LTC_4 synthase activity and that was constitutively present in THP-1 cells without further differentiation, as was LTC_4 synthase activity.

The N-terminal sequence of human LTC_4 synthase was composed predominantly of hydrophobic amino acids, consistent with the membrane-bound localization of the enzyme. It contained an N-terminal methionine residue, indicating that human LTC_4 synthase is probably not proteolytically processed during its biogenesis *in vivo*. Most interestingly, a consensus sequence for protein kinase C phosphorylation was present, suggesting that human LTC_4 synthase may be a phosphoregulated enzyme. Phosphoregulation of LTC_4 synthase may constitute a potentially important controlling mechanism that may substantially affect cysteinyl LT production in several inflammatory disease states. Preliminary results in our laboratories using HL-60 cells have demonstrated that phorbol 12-myristate 13-acetate, an activator of protein kinase C, abolished cysteinyl LT production in ionophore-challenged HL-60 cells, whereas inhibition could be prevented by the presence of the protein kinase C inhibitor staurosporine (S. Kargman, J.P.V., J. F. Evans, and D.W.N., unpublished data).

In summary, human LTC_4 synthase, a homodimeric glutathione *S*-transferase composed of two identical 18-kDa subunits, has been purified to homogeneity. LTC_4 synthase is responsible for the production of LTC_4 and may, therefore, be a key controlling enzyme in the biosynthesis of inflammatory LT mediators.

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