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Interconversion of leukotrienes catalyzed by purified γ-glutamyl transpeptidase: Concomitant formation of leukotriene D4 and γ-glutamyl amino acids

(γ-glutamyl leukotrienes/glutathione/dipeptidase/aminopeptidase M)

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ABSTRACT **The reversible conversion of leukotriene C4 to leukotriene D4 and of the latter to leukotriene E4 were studied with highly purified homogeneous preparations of γ-glutamyl transpeptidase, dipeptidase, and aminopeptidase M. The conversion of leukotriene C4 to leukotriene D4, catalyzed by γ-glutamyl transpeptidase, is significantly more rapid when carried out in the presence of an amino acid mixture closely approximating that found in blood plasma and is accompanied by γ-glutamyl amino acid formation. Because γ-glutamyl transpeptidase is bound to the external surface of cell membranes and thus is readily accessible to plasma amino acids, it appears that conversion of leukotriene C4 to leukotriene D4 under physiological conditions is coupled with the formation of γ-glutamyl amino acids. The apparent K_m value for leukotriene C4 in this reaction is about 6×10^{-6} M, a value close to that found for glutathione. Conversion of leukotriene D4 to leukotriene C4 is effectively catalyzed by γ-glutamyl transpeptidase in the presence of relatively low concentrations of glutathione. The conversion of leukotriene D4 to leukotriene E4 is catalyzed much more rapidly by renal dipeptidase than by renal aminopeptidase M. Incubation of leukotriene E4 with γ-glutamyl transpeptidase and glutathione leads to formation of a compound with the properties of γ-glutamyl leukotriene E4; this reaction is analogous to that shown previously in which γ-glutamyl cystine is formed by transpeptidation between glutathione and cystine.**

Studies carried out about a century ago showed that administration of chlorobenzene or of bromobenzene to dogs leads to urinary excretion of mercapturic acids (*N*-acetyl *S*-substituted cysteine derivatives) (1, 2). Subsequent work established that many "foreign" compounds can react with glutathione in a process often mediated by glutathione *S*-transferases to form *S*-substituted glutathiones (3–6). The γ-glutamyl and glycine moieties of these are enzymatically cleaved, and the resulting *S*-substituted cysteines are *N*-acetylated. It was also found that *S*-substituted glutathione derivatives are formed from endogenous compounds and thus are involved in the metabolism of estrogens (7–11), prostaglandins (12, 13), and melanin (14). Recent studies on slow reacting substances associated with anaphylaxis and related phenomena (15–17) have led to the isolation and synthesis of several such compounds, including 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene C4) (15–28). This compound is formed by a pathway of metabolism in which leukotriene A4, an epoxide derived from arachidonic acid, reacts with glutathione to form leukotriene C4. Removal of the γ-glutamyl moiety of leukotriene C4 yields leukotriene D4; cleavage of the glycine moiety of the latter compound leads to formation of leukotriene E4. These transformations have been examined with tissue preparations and commercially available enzyme preparations.

In the present work, in which very highly purified enzyme preparations isolated in the course of our studies on glutathione metabolism (29, 30) were used, we have examined the reversible conversion of leukotriene C4 to leukotriene D4 catalyzed by γ-glutamyl transpeptidase. We also have studied the enzymatic conversion of leukotriene D4 to leukotriene E4 and the formation of a compound with the properties of γ-glutamyl leukotriene E4 catalyzed by γ-glutamyl transpeptidase.

EXPERIMENTAL PROCEDURES

The amino acids, glutathione, L-γ-glutamyl-*p*-nitroanilide, glycylglycine, *tris*(hydroxymethyl) aminomethane, and 2-methylimidazole (recrystallized from ethyl acetate) were obtained from Sigma. L-[U-^{14}C]Cystine was obtained from Schwarz/Mann and [U-^{14}C]glutamyl-labeled glutathione was obtained from New England Nuclear. γ-Glutamyl transpeptidase, which was purified from rat kidney (31), was devoid of dipeptidase activity and exhibited a specific activity of 425 units (μmol/min per mg) when assayed at 37°C in a reaction mixture containing 2.5 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 150 mM NaCl, and 25 mM 2-methylimidazole (pH 8.0) (32). Purified rat kidney brush border dipeptidase and aminopeptidase M were provided by S. S. Tate. Kozak and Tate (33) found that these enzymes exhibited activities of 896 μmol/min per mg and 12.9 μmol/min per mg when assayed in 0.1 M Tris·HCl (pH 8.0) and 2.5 mM L-alanylglycine at 37°C.

Leukotrienes C4, D4, and E4 were provided by J. Rokach of Merck Frosst Laboratories (Pointe-Claire/Dorval, PQ, Canada). The concentrations of the stock solutions of leukotrienes were determined by measuring the absorbance at 280 nm and assuming a molar extinction coefficient of 40,000 cm^{-1} M^{-1} (18, 19).

The amino acid mixture was that used previously (32), which closely approximates the concentrations found in blood plasma. The amino acids (final concentrations in the reaction mixtures are given in parentheses) were: L-alanine (0.5 mM), L-arginine (0.12 mM), L-asparagine (0.25 mM), L-aspartate (0.02 mM), L-cystine (0.04 mM), L-glutamate (0.04 mM), L-glutamine (0.4 mM), L-histidine (0.08 mM), L-isoleucine (0.06 mM), L-leucine (0.15 mM), L-lysine (0.2 mM), L-methionine (0.04 mM), L-phenylalanine (0.13 mM), L-serine (0.19 mM), L-threonine (0.2 mM), L-tryptophan (0.05 mM), L-tyrosine (0.08 mM), L-valine (0.2 mM), and glycine (0.4 mM) (total amino acid concentrations, 3.15 mM). L-Glutamine was added last, just prior to addition of enzyme. The reactions were initiated by adding enzyme and were quenched after incubation at 37°C by adding 0.5 vol of ice-cold 225 mM acetic acid and freezing in a bath of dry ice/acetone. The samples were thawed to room temperature just prior to analysis.

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Formation of γ-glutamyl [^{14}C]cystine was determined after separation of γ-glutamyl [^{14}C]cystine from [^{14}C]cystine. The quenched reaction mixtures were immediately applied to a Bio-Rad AG1-X8-acetate column (3.5 × 0.5 cm). The columns were eluted with 4 ml of water, followed successively by 5 ml each of 20 mM acetic acid, 700 mM acetic acid, 1.8 M acetic acid, and 1.8 M ammonium acetate (pH 7.0). Fractions of 1 ml were collected directly into scintillation vials containing 10 ml of Dimiscint (National Diagnostics, Somerville, NJ). Under these conditions, most of the cystine is eluted with water and the remainder with 20 mM acetic acid; 80% of the γ-glutamylcystine is eluted with 700 mM acetic acid and the remainder with 1.8 M acetic acid (traces of it are eluted with ammonium acetate).

In the experiments with dipeptidase and aminopeptidase M and in the studies on conversion of leukotriene D4 to leukotriene C4, leukotriene C4 was initially hydrolyzed to leukotriene D4 by adding γ-glutamyl transpeptidase. Then, dipeptidase, aminopeptidase M, or a stock solution of freshly prepared glutathione, was added to initiate the reaction.

Leukotrienes were determined by high-performance liquid chromatography (34) with a Waters C-18 μBondapak column (3.9 mm × 30 cm), isocratic elution [65% methanol/35% water/0.01% acetic acid (vol/vol); pH adjusted with NH_4OH to 5.7] with a flow rate of 1 ml/min Waters M-6000 A pump, Waters Intelligent Sample Processor, data module, system controller, and ultraviolet detector (280 nm; full scale, 0.01). The retention times for leukotrienes C4, D4, and E4 were 13, 22, and 25 min, respectively. Small shoulders found on the leukotriene peaks are thought to reflect the occurrence of *cis–trans* isomerism. Apparent impurities (about 5% of main peaks) were found in the preparations of leukotrienes C4, D4, and E4 at 9, 14, and 17 min, respectively. Because the retention times varied about 10–15% with different batches of eluants, appropriate standard compounds were included in each series of experiments.

RESULTS

When leukotriene C4 was incubated at 37°C with purified γ-glutamyl transpeptidase in reaction mixtures (144 μl) containing enzyme (0.0078 unit), leukotriene C4 (2.11, 4.23, 9.40, 16.4, or 34.0 μM), and sodium phosphate buffer (40 mM; pH 7.4), there was rapid disappearance of leukotriene C4 and equivalent formation of leukotriene D4. The apparent K_m value for leukotriene C was 5.9×10^{-6} M. These values are in close agreement with those found for glutathione (K_m, 5.7×10^{-6} M) and γ-glutamyl-*p*-nitroanilide (K_m, 5.8×10^{-6} M) determined under the same conditions. The rate of conversion of leukotriene C4 to leukotriene D4 in reaction mixtures (144 μl) containing enzyme (0.0078 unit) and leukotriene C (33.3 μM) increased from 5.06 nmol/min to 11.7 nmol/min when 4.5 mM glycylglycine was present.

When the reaction was carried out in the presence of an amino acid mixture closely approximating the concentration of amino acids in blood plasma (see *Methods* and ref. 32), the rate of conversion of leukotriene C4 to leukotriene D4 was also increased substantially (Fig. 1). Previous studies in which glutathione was incubated with amino acids and the enzyme under similar conditions showed that a significant fraction of the glutathione utilized participated in transpeptidation (32). A similar result was obtained in the present studies in which conversion of leukotriene C4 to leukotriene D4 was carried out in the presence of L-[^{14}C]cystine; the reaction mixtures (144 μl) contained leukotriene C4 (17.6 μM), L-[^{14}C]cystine (40 μM; 89,000 cpm), γ-glutamyl transpeptidase (0.000055 unit), and sodium phosphate buffer (40 mM; pH 7.4). The rate of formation of leukotriene D4 was 0.0867 nmol/min, and that of γ-glutamyl-[^{14}C]cystine was 0.045 nmol/min. Thus, under these conditions,

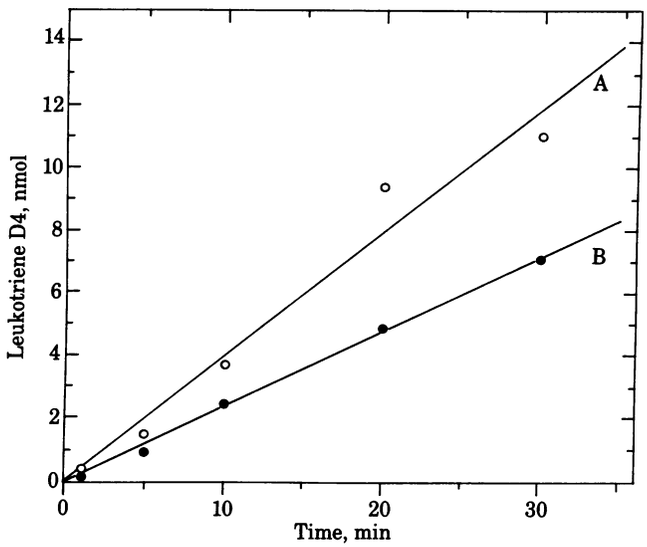

FIG. 1. Conversion of leukotriene C4 to leukotriene D4 in the presence (line A) and absence (line B) of a mixture of amino acids approximating that found in blood plasma. The reaction mixtures (144 μl) contained initially γ-glutamyl transpeptidase (0.000333 unit), leukotriene C4 (31.1 μM), amino acids (see *Methods*; 3.15 mM), and sodium phosphate buffer (40 mM; pH 7.4).

a substantial fraction of the leukotriene C4 used participated in transpeptidation. This result is in close agreement with analogous studies on glutathione (32).

Conversion of leukotriene D4 to leukotriene C4 was demonstrated in reaction mixtures (144 μl) containing γ-glutamyl transpeptidase (0.017 unit), leukotriene C4 (24.6 μM), and sodium phosphate buffer (40 mM; pH 7.4). After incubation at 37°C for 30 min, analysis of a sample indicated 100% conversion of leukotriene C4 to leukotriene D4. Another sample was treated with glutathione to yield a final concentration of 0.5 mM, and incubation was continued for an additional 30 min. Analysis of this sample revealed formation of 0.43 nmol of leukotriene C4; this is equivalent to conversion of 12% of the leukotriene D4 present to leukotriene C4.

The conversion of leukotriene D4 to leukotriene E4 was examined in the presence of highly purified preparations of rat kidney dipeptidase and aminopeptidase M (33). These homogeneous enzyme preparations were found by Kozak and Tate (33) to hydrolyze L-alanylglycine at rates of 896 and 12.9 μmol/min per mg, respectively. We examined the hydrolysis of leukotriene D4 to leukotriene E4 in reaction mixtures (37 μl) containing 50.1 μM and 77.6 μM leukotriene D4, aminopeptidase (0.12 unit) or dipeptidase (0.0039 unit), and Tris·HCl buffer (40 mM; pH 7.4). The rates of leukotriene E4 formation catalyzed by dipeptidase were 7.03 and 16.4 μmol/min per mg, whereas the corresponding values of hydrolysis observed with aminopeptidase were 1.3 and 0.79 nmol/min per mg. Thus, leukotriene D4 is a much better substrate of the dipeptidase than of the aminopeptidase; however, it is split at only about 1–2% of the rate observed for cleavage of L-alanylglycine. On the other hand, the aminopeptidase splits leukotriene D4 at about 0.01% of the rate found with L-alanylglycine.

Enzymatic conversion of leukotriene E4 by γ-glutamyl transpeptidase to a product exhibiting the properties of γ-glutamyl leukotriene E4 was accomplished by incubating leukotriene E4 with γ-glutamyl transpeptidase and [U-^{14}C]glutamyl-labeled glutathione in reaction mixtures (200 μl) containing leukotriene E4 (28.8 μM), enzyme (0.12 unit), glutathione (0.67 mM), and sodium phosphate buffer (40 mM; pH 7.4). The mixture was

incubated at 37°C for 60 min, and the reaction was then quenched as described. Analysis was carried out in the usual way by high-performance liquid chromatography and by determining the radioactivity present in the individual fractions eluted from the column. An absorbance peak appearing at 8.03 min (Fig. 2*A*) was observed in an elution position consistent with that expected for γ-glutamyl leukotriene E4. In accord with such expectation, this absorbance peak corresponded exactly with the only peak of radioactivity found on the chromatogram (Fig. 2, below). The calculated amount of γ-glutamyl leukotriene E4 formed under these conditions, based on the radioactivity found, was 0.4 ± 0.1 nmol; this value is in close agreement with that obtained from the absorbance data, if one assumes that the absorbance of γ-glutamyl leukotriene E4 is the same as that of leukotriene E4.

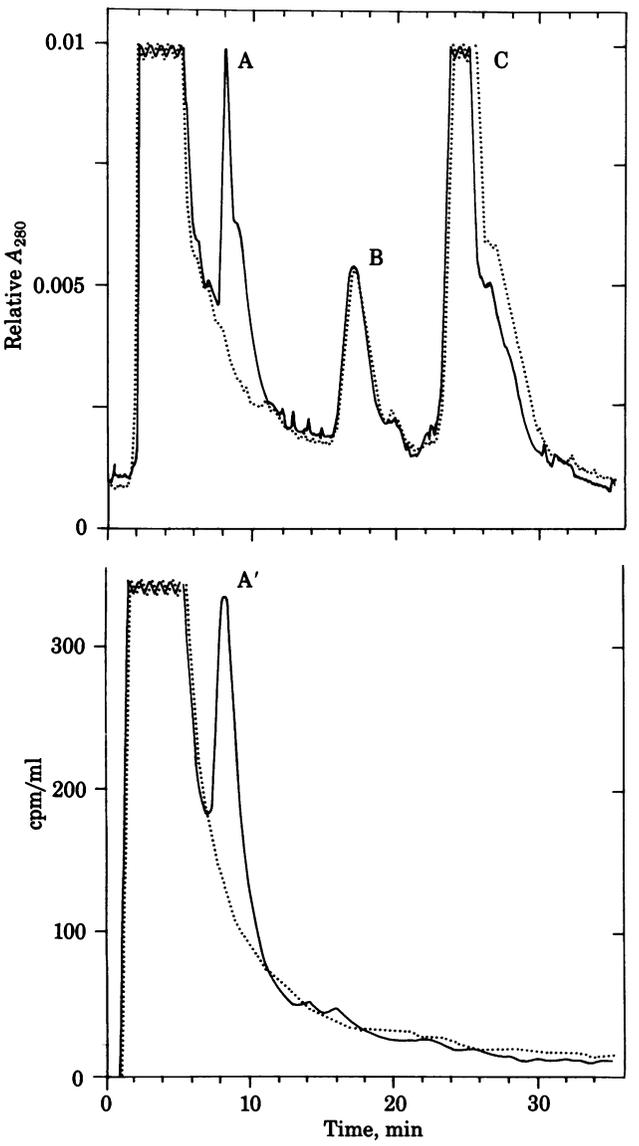

FIG. 2. Formation of γ-glutamyl leukotriene E4. The reaction mixtures (200 μl) contained initially γ-glutamyl transpeptidase (0.12 unit), leukotriene E4 (29.7 μM), [U-^{14}C]glutamyl-labeled glutathione (0.67 mM; 134,000 cpm), dithiothreitol (0.67 mM), and sodium phosphate buffer (40 mM; pH 7.4). After incubation at 37°C for 30 min, the reaction was treated with acetic acid and placed in a dry ice/acetone bath (see *Methods*). Portions were chromatographed, and the absorbance at 280 nm (*Upper*) was determined. Fractions of 1 ml (per min) were collected for determination of radioactivity (*Lower*). ·····, Control in which enzyme was added after treatment with acetic acid. Peaks: A, γ-Glutamyl leukotriene E4; B, an impurity or degradation product eluting at 12 min; C, leukotriene E4.

DISCUSSION

The present findings, which are in accord with the pathway of metabolism of the leukotrienes indicated by previous studies, suggest several points that may be of significance in relation to the physiological transformations of these interesting new compounds.

(*i*) We found that the apparent K_m value for leukotriene C4 with γ-glutamyl transpeptidase is about 6×10^{-6} M or about the same as that found for glutathione. This observation is consistent with the view that leukotriene C4 is a physiologically significant substrate of γ-glutamyl transpeptidase and that it may compete with glutathione for the enzyme (26, 27). The high affinity of γ-glutamyl transpeptidase for leukotriene compounds indicates that this enzyme could function in several types of leukotriene transformations. The wide distribution of the enzyme in mammalian tissues suggests that such reactions may take place in many locations. These factors would appear to complicate studies on the relative biological effectiveness of different leukotriene compounds.

(*ii*) There is considerable evidence that γ-glutamyl transpeptidase is membrane-bound and that it is localized on the external surface of cell membranes in a variety of anatomical locations. Thus, it is likely that removal of the γ-glutamyl moiety of glutathione conjugates such as leukotriene C4 takes place extracellularly in the presence of amino acids. The present studies show that the conversion of leukotriene C4 to leukotriene D4 is accelerated by the presence of a mixture of amino acids comparable to that which occurs under physiological conditions and that this conversion is associated with γ-glutamyl amino acid formation. The findings are consistent with a mechanism involving γ-glutamyl-enzyme formation (29), and indicate that γ-glutamyl-enzyme formation is not rate limiting in the interaction of the enzyme with leukotriene C4.

(*iii*) Because γ-glutamyl transpeptidase catalyzes a freely reversible reaction, and because the *in vivo* concentrations of glutathione are substantial, it is quite possible that γ-glutamyl transpeptidase can catalyze *in vivo* conversion of leukotriene D4 to leukotriene C4.

(*iv*) The present findings indicate that highly purified renal dipeptidase catalyzes effective conversion of leukotriene D4 to leukotriene E4. This enzyme, or a similar activity present in other tissues may be of significance in the formation of leukotriene E4. In contrast, renal aminopeptidase is much less active in catalyzing this transformation.

(*v*) The data presented here indicate that leukotriene E4 may be converted to γ-glutamyl leukotriene E4.* This reaction, which is analogous to the conversion of cystine to γ-glutamyl cystine previously shown in this laboratory to be catalyzed *in vivo* by γ-glutamyl transpeptidase (35), may also take place *in vivo*. It would be of interest to determine whether this compound is present in biological materials.

(*vi*) It is well known that γ-glutamyl transpeptidase can catalyze the formation (by "autotranspeptidation") of products containing more than one γ-glutamyl group; thus, in one study, a product containing four γ-glutamyl groups was obtained (36). It is conceivable that such compounds may be formed from leukotrienes of the C and E types; such reactions, which would be favored by the relatively high glutathione concentrations found

* Independent evidence for the formation of γ-glutamyl leukotriene E has been obtained by K. Bernström and S. Hammarström (personal communication).

in vivo, might account for additional products of leukotriene metabolism.

The reactions observed here are analogous to the corresponding reactions of glutathione metabolism and to those involved in the metabolism of *S*-substituted glutathione conjugates formed in exogeneous (1–6) and endogeneous (7–14) metabolism previously studied. A futher analogy relates to the finding (30, 37–42) that glutathione is transported out of many cells at significant rates. It appears likely that cells also export glutathione conjugates, possibly by the same mechanism involved in the export of glutathione. Although the general pathway of *S*-substituted glutathione conjugates has been extensively studied, relatively little consideration has been given to the cellular locations at which the various reactions occur. Because γ-glutamyl transpeptidase and dipeptidase are membrane bound, and because the glutathione *S*-transferases are intracellular, it appears that the formation of glutathione conjugates takes place intracellularly and that these are translocated out of the cell, where additional metabolism leading to the formation of *S*-substituted cysteine conjugates occurs. Subsequent transport of *S*-substituted cysteine conjugates into the cell must then occur, followed by *N*-acetylation and possibly other reactions. The possibility that the γ-glutamyl derivatives of *S*-substituted cysteine conjugates are involved in such transport should be considered because there is substantial evidence for the transport of γ-glutamyl amino acids.

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