Transformation of leukotriene A_4 methyl ester to leukotriene C_4 monomethyl ester by cytosolic rat glutathione transferases

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Six major basic cytosolic glutathione transferases from rat liver catalyzed the conversion of leukotriene A_4 methyl ester to the corresponding leukotriene C_4 monomethyl ester. Glutathione transferase 4-4, the most active among these enzymes, had a V_{max} of 615 nmol · min^{-1} · mg protein $^{-1}$ at 30°C in the presence of 5 mM glutathione. It was followed in efficiency by transferase 3-4 which had a V_{max} of 160 nmol · min^{-1} · mg^{-1} under the same conditions. Transferases 1-1, 1-2, 2-2 and 3-3 had at least 30 times lower V_{max} values than transferase 4-4.

Glutathione transferase *Leukotriene* *Isoenzyme* *Kinetics* *Slow reacting substance of anaphylaxis*

1. INTRODUCTION

The cysteine-containing leukotrienes, LTC_4, LTD_4, and LTE_4 constitute the so-called 'slow reacting substance of anaphylaxis' (SRS-A), a presumed mediator of allergic and anaphylactic reactions [1]. These compounds are formed by conjugation of glutathione with an epoxy acid derived from arachidonic acid (LTA_4) to give LTC_4, followed by successive elimination of glutamic acid to give LTD_4 and glycine to give LTE_4. A recent report has dealt with the conversion of LTA_4 free acid to LTC_4 by microsomal enzymes from rat basophilic leukemia cells and from rat liver [2]. This investigation was undertaken to evaluate the possible contribution of the abundant hepatic cytosolic glutatione transferases in the biosynthesis of leukotrienes. This group of enzymes catalyzes the conjugation of glutathione with a wide variety of electrophilic compounds, including epoxides [3].

Abbreviations: HPLC, high-performance liquid chromatography; LTA_4, leukotriene A_4 (*trans*-5(*S*),6(*S*)-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid); LTC_4, leukotriene C_4 (5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11, 14-*cis*-eicosatetraenoic acid); LTD_4, leukotriene D_4 (5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid); LTE_4, leukotriene E_4 (5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid)

2. MATERIALS AND METHODS

2.1. *Chemicals*

LTA_4 methyl ester was a generous gift of J. Rokach, Merck-Frosst, Canada. [14,15-$^3H_2$] LTA_4 methyl ester was purchased from New England Nuclear.

2.2. *Enzymes*

Glutathione transferases were isolated from rat liver cytosol by use of affinity chromatography on *S*-hexyl glutathione Sepharose and chromatofocusing essentially as described earlier [4]. Chromatofocusing was performed on a Mono P column using the fast protein liquid chromatography system of Pharmacia. Transferase 2-2 was further chromatographed on CM-Sepharose to obtain homogeneity, whereas transferases 1-1, 1-2, 3-3,

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3-4 and 4-4 were pure after chromatofocusing. A new nomenclature for rat glutathione transferases [5] has been adopted; transferases L_2, BL, B_2 A_2, AC, and C_2 [4] are now called 1-1, 1-2, 2-2, 3-3, 3-4, and 4-4, respectively.

Soybean lipoxygenase type II and γ-glutamyl transpeptidase from porcine kidney were purchased from Sigma.

2.3. *Hydrolysis of leukotriene C_4 methyl ester*

LTC_4(C-1) monomethyl ester (1–5 nmol) was treated with 0.2 ml of 0.3 M NaOH in 50% aqueous methanol for 30 min at 22°C.

2.4. *Incubation conditions*

Stock solutions of ^3H-labeled LTA_4 methyl ester were prepared in hexane containing 1% triethylamine by mixing appropriate amounts of [14,15-$^3H_2$]LTA_4 methyl ester and unlabeled compound. One solution was 1.2 mM with a specific radioactivity of 8.3 Ci/mol and a second solution was 0.0122 mM with a specific radioactivity of 820 Ci/mol. The stock solutions were kept at -70°C. Before use of the stored material, the solvent was removed with a stream of argon and the original volume restored by addition of ethanol.

The incubation mixtures (0.104–0.110 ml) contained glutathione transferase (varying amounts), 25 mM potassium phosphate buffer (pH 7.0) and 5 mM glutathione. The mixtures were kept at 37°C (30°C in kinetic experiments) for 1 min prior to and 1 min after the addition of glutathione. Reactions were started by addition of LTA_4 methyl ester (final concentration: 1–49 μM) and terminated after 15 min (1 min in kinetic experiments) by addition of 4 vols of ice-cold ethanol.

2.5. *Analytical procedures*

The total reaction mixtures were analyzed by reverse-phase HPLC using columns of C_{18} Nucleosil (Machery Nagel Co; column dimensions 4.6×250 mm, 5 μm particles) and with methanol/water (7:3, v/v) plus 0.07% acetic acid/0.03% phosphoric acid, adjusted to pH 5.4 with NH_4OH as mobile phase (flow rate: 1 ml/min).

Alternatively, incubation mixtures were diluted with 1 ml water (after the addition of ethanol) and extracted 3 times with ethyl acetate. The tritium contents in the pooled organic phases and in the remaining aqueous phase were determined by liquid scintillation counting in a Packard Tri Carb model 3385 instrument equipped with automatic external standardization. To correct for non-enzymatic reactions, blanks without enzyme were processed in the same way. The amount of tritium in the aqueous phase, corrected for non-enzymatic contribution, was used as a measure of the formation of LTC_4 monomethyl ester. HPLC analyses of aqueous phase confirmed that this was a valid assumption.

2.6. *Conversions with soybean lipoxygenase and γ-glutamyl transpeptidase*

The products formed from LTA_4 methyl ester (1 μM) in Tyrode's buffer were treated with soybean lipoxygenase (0.01 mg/ml). The reactions were monitored by UV spectrometry [7]. Alternatively, the products were treated with γ-glutamyl transpeptidase and the transformation monitored by HPLC [6].

3. RESULTS

3.1. *Formation of LTC_4 (C-1) monomethyl ester by cytosolic rat glutathione transferases*

Incubation of 25 μg rat cytosolic glutathione

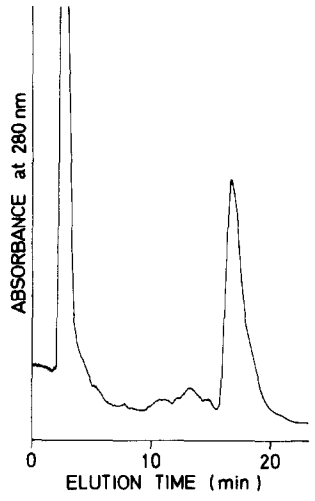

Fig. 1. Reverse-phase HPLC of products obtained from LTA_4 methyl ester (44 μM) and glutathione (5 mM) in the presence of glutathione transferase 4-4 from rat liver cytosol (0.23 mg/ml) after 15 min at 37°C.

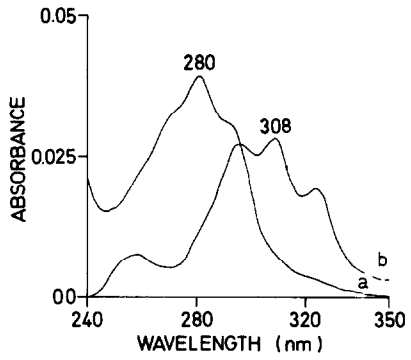

Fig. 2. UV spectrum of the main product in fig. 1 after mild alkaline hydrolysis (a) before and (b) after treatment with soybean lipoxygenase (0.01 mg/ml) for 30 min at 20°C.

transferase 4-4 for 15 min with 4.8 nmol tritium-labeled LTA_4 methyl ester and 500 nmol glutathione yielded a tritium-labeled product which emerged after 18 min on reverse-phase HPLC (fig. 1). The UV spectrum of this compound showed a λ_{max} at 280 nm and shoulders at 270 and 292 nm as previously reported for LTC_4 [7]. Treatment of this product with soybean lipoxygenase shifted the λ_{max} to 308 nm (fig. 2). After mild alkaline hydrolysis, the product cochromatographed with authentic LTC_4 on reverse-phase HPLC (fig. 3). Incubation of the product with γ-glutamyl transpeptidase followed by mild alkaline hydrolysis gave rise to LTD_4 as judged by cochromatography on HPLC with the synthetic reference compound.

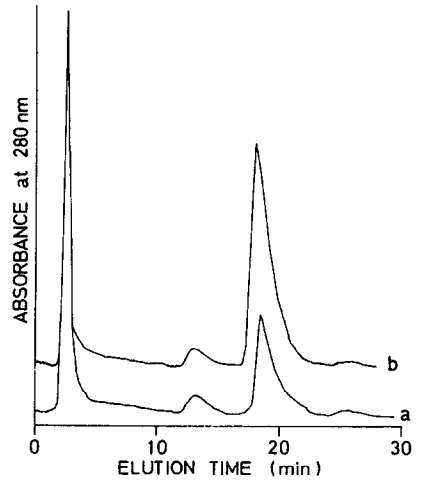

Fig. 3. Reverse-phase HPLC of the product in fig. 1 after mild alkaline hydrolysis (a) before and (b) after addition of synthetic LTC_4.

Incubation of LTA_4 methyl ester with rat liver cytosolic transferases 1-1 (37 μg), 1-2 (37 μg), 2-2 (24 μg), 3-3 (33 μg), or 3-4 (49 μg) in each case yielded tritium-labeled products with similar chromatographic and UV absorbing properties as the product formed by transferase 4-4. After mild alkaline hydrolysis, these products cochromatographed with synthetic LTC_4 on HPLC.

3.2. *Specific activities of cytosolic liver glutathione transferases with LTA_4 methyl ester as substrate*

The different basic rat liver cytosolic glutathione transferases were incubated with 12 μM [^{3}H]LTA_4 methyl ester at 30°C for 1 min. The conversion to LTC_4 methyl ester was determined by ethyl acetate extraction and the specific enzyme activities were calculated from the radioactivity remaining in the aqueous phase in relation to the total radioactivity recovered (table 1). Glutathione transferase 4-4 had the highest specific activity and transferase 3-4 had approximately 3-times lower specific activity. Transferases 1-1, 1-2, 3-3, and 2-2 had specific activities which were 7.5, 5.5, 2.5 and 0.8% those of transferase 4-4, respectively. For comparison, the specific activities using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate are also given in table 1.

3.3. *Kinetics of the reaction with LTA_4 methyl ester catalyzed by cytosolic glutathione transferases*

The kinetics of LTC_4 monomethyl ester formation were studied as a function of LTA_4 methyl ester concentration at 5 mM glutathione concentration. The rate of formation of LTC_4 monomethyl ester was constant for at least 1 min under the conditions used and was proportional to the enzyme concentration (not shown).

The kinetic parameters for the conversion of LTA_4 methyl ester to LTC_4 monomethyl ester by rat liver cytosolic glutathione transferases are shown in table 1. The K_m values for the different isoenzymes ranged from 2.3 to 15 μM and the V_{max} values from 17 to 615 nmol · min^{-1} · mg protein^{-1}. Transferase 4-4 had the highest V_{max}. This enzyme is a homodimer of subunit '4'. The heterodimeric

Table 1

Specific activities with LTA_4 methyl ester and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates and kinetic properties with LTA_4 methyl ester as substrate of cytosolic rat glutathione transferases

Glutathione transferase	Specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)		V_{max} ($nmol \cdot min^{-1} \cdot mg^{-1}$)	K_m (μM)
	LTA_4 methyl ester[a]	CDNB[b]		
1-1	7.6 ± 2.7	33,000	17	2.3
1-2	5.6 ± 2.8	28,000	17	3.3
2-2	0.83 ± 0.25	19,000	n.d.	n.d.
3-3	2.5 ± 1.1	38,000	22	9.2
3-4	32 ± 22	28,000	162 ± 20[c]	15 ± 0.3
4-4	102 ± 18	18,000	615 ± 15[c]	11 ± 4

[a] Mean values ± S.D. from 3 experiments with single or duplicate analyses
[b] Data from [4]
[c] n = 2

n.d. = not determined

transferase 3-4, which contains one subunit '3' and one subunit '4' had a V_{max} somewhat over 25% that of transferase 4-4. The other transferases (the homodimeric transferases 3-3 and 1-1 and the heterodimeric transferase 1-2) had V_{max} values of approx. 20 $nmol \cdot min^{-1} \cdot mg^{-1}$ (3% that of 4-4). Subunit '4' thus appears to differ from subunits '3', '2', and '1' by its efficacy to convert LTA_4 methyl ester to LTC_4 monomethyl ester.

4. DISCUSSION

The glutathione transferases are a group of enzymes catalyzing a variety of reactions involving glutathione [3,8,9]. Their main function, according to current concepts, is to detoxify and accelerate the excretion of certain xenobiotic compounds.

The demonstration that 'slow reacting substance of anaphylaxis' (SRS-A) is formed from arachidonic acid and glutathione [7,10] in a pathway involving hydroperoxy- and epoxy-acid (LTA_4) intermediates [11-13] suggested that leukotrienes might serve as endogenous substrates for glutathione transferases. Here, we demonstrate that 6 basic glutathione transferases from rat liver cytosol [4] convert LTA_4 methyl ester to LTC_4 (C-1) monomethyl ester in the presence of reduced glutathione.

The product formed by glutathione transferase 4-4 was characterized by UV spectroscopy, treatment with soybean lipoxygenase and γ-glutamyl transpeptidase and cochromatography with synthetic LTC_4 after initial removal of the methyl ester group by mild alkaline hydrolysis. These methods of characterization discriminate between regio- and stereoisomers of LTC_4 (cf. [14]). It is therefore clear that the product formed from LTA_4 methyl ester by transferase 4-4 has the structure methyl 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoate (LTC_4 (C-1) monomethyl ester). Thus, the methyl ester of the

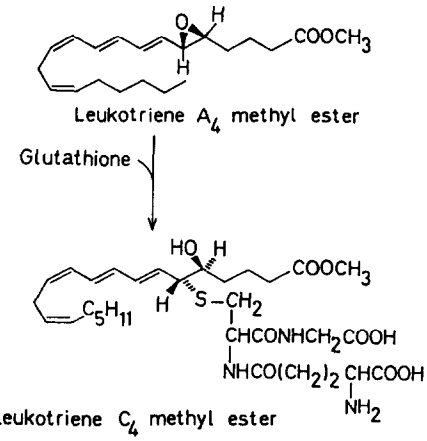

Fig. 4. Conversion of LTA_4 methyl ester to LTC_4 (C-1) monomethyl ester by cytosolic glutathione transferases.

naturally occurring isomer of LTC_4 was formed and there was no evidence for formation of monomethyl esters of other isomers including 11-*trans* LTC_4 and 5,12-LTC_4. The reaction is therefore a nucleophilic substitution of the oxygen at C-6 in LTA_4 methyl ester by the thiolate anion of glutathione proceeding with inversion of the configuration at C-6. The products formed by the 5 remaining glutathione transferases from rat liver cytosol were identified as LTC_4 after mild alkaline hydrolysis and cochromatography on reverse-phase HPLC with synthetic reference compound.

The pattern of the relative catalytic activities of the cytosolic glutathione transferases reflected their respective subunit compositions as earlier demonstrated with other substrates [4]. V_{max} values for the reactions with leukotriene A_4 methyl ester ranged from 17 to 615 $nmol \cdot min^{-1} \cdot mg^{-1}$. Transferase 4-4 had the highest (615) and transferase 3-4 the second highest V_{max} value (162). K_m values were ~ 10 μM for transferases containing subunits 3 and/or 4 and ~ 3 μM for transferases composed of subunits 1 and/or 2. Thus, the transferases with highest apparent 'affinity' for LTA_4 had the lowest capacity to transform it into LTC_4 monomethyl ester and *vice versa*.

Although LTA_4 methyl ester was efficiently converted to LTC_4 monomethyl ester (fig. 4), particularly by transferases 4-4 and 3-4, preliminary results indicate that the free acid is converted to LTC_4. Further studies regarding the kinetics of the latter reaction are needed to compare the catalytic efficiencies of cytosolic rat glutathione transferases with those of microsomal enzymes from murine mastocytoma cells and rat basophilic leukemia cells which catalyze the same reaction (Söderström, M. et al., unpublished). Such studies are in progress.

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