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Leukotriene C synthase in mouse mastocytoma cells

An enzyme distinct from cytosolic and microsomal glutathione transferases

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Leukotriene C_4 synthesis was studied in preparations from mouse mastocytoma cells. Enzymic conjugation of leukotriene A_4 with glutathione was catalysed by both the cytosol and the microsomal fraction. The specific activity of the microsomal fraction (7.8 nmol/min per mg of protein) was 17 times that of the cytosol fraction. The cytosol fraction of the mastocytoma cells contained two glutathione transferases, which were purified to homogeneity and characterized. A microsomal glutathione transferase was purified from mouse liver; this enzyme was shown by immunoblot analysis to be present in the mastocytoma microsomal fraction at a concentration one-tenth or less of that in the liver microsomal fraction. Both the cytosolic and the microsomal glutathione transferases in the mastocytoma cells were identified with enzymes previously characterized, by determining specific activities with various substrates, sensitivities to inhibitors, reactions with antibodies, and physical properties. The purified microsomal glutathione transferase from liver was inactive with leukotriene A_4 or its methyl ester as substrate. The cytosolic enzymes displayed activity with leukotriene A_4, but their specific activities and intracellular concentrations were too low to account for the leukotriene C_4 formation in the mastocytoma cells. The microsomal fraction of the cells contained an enzyme distinguishable by various criteria from the previously studied glutathione transferases. This membrane-bound enzyme, leukotriene C synthase (leukotriene A_4:glutathione *S*-leukotrienyltransferase), appears to carry the main responsibility for the biosynthesis of leukotriene C_4.

INTRODUCTION

The cysteine-containing leukotrienes C_4, D_4 and E_4 are a group of biologically active compounds with smooth-muscle-stimulating and oedema-inducing properties [1–4]. These compounds are presumed to mediate anaphylactic and allergic reactions. Leukotriene C_4 is formed by conjugation of the epoxy group of leukotriene A_4 with glutathione [5–8]. Elimination of glutamic acid, catalysed by γ-glutamyltransferase, gives leukotriene D_4; further elimination of glycine, catalysed by a dipeptidase, gives leukotriene E_4, and finally, acetylation of the amino group of leukotriene E_4 by an *N*-acetyltransferase gives *N*-acetyl-leukotriene E_4 [9–12]. The nature of the catalytic activity responsible for the conversion of leukotriene A_4 into leukotriene C_4, namely leukotriene C synthase, has not been established. Reports from three groups have shown that leukotriene C_4 formation from leukotriene A_4 in rat basophilic leukaemia cells is catalysed by a membrane-bound enzyme [8,13,14]. Cytosolic glutathione transferases have also been reported to catalyse the conversion of leukotriene A_4 into leukotriene C_4 [15–17], but their importance relative to that of the membrane-bound activity has not been established.

The formation of leukotriene C_4 is especially high in mouse mastocytoma cells, the source from which leukotriene C_4 was first isolated [18]. The present investigation was carried out to determine whether the formation of leukotriene C_4 in these cells is attributable to a specific leukotriene C synthase or to cytosolic or microsomal glutathione transferases. Furthermore, parameters such as substrate specificities, IC_{50} values, isoelectric points and apparent subunit M_r values were determined in order to obtain criteria for discrimination between the cytosolic and the microsomal glutathione transferase, as well as for the microsomal enzyme leukotriene C synthase.

MATERIALS AND METHODS

Materials

Leukotriene A_4 methyl ester was generously given by J. Rokach, Merck–Frosst Inc., Point-Claire, Dorval, Que., Canada. [14,15-$^3H_2$]Leukotriene A_4 methyl ester was purchased from New England Nuclear. Leukotriene A_4 was obtained by hydrolysis of the methyl ester with LiOH in tetrahydrofuran as previously described [15]. Before use, the hydrolysis mixture was evaporated to dryness under a stream of argon and dissolved in ethanol. *S*-Hexylglutathione–Sepharose 6B was prepared as described by Mannervik & Guthenberg [19]. Chromatography materials were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were standard commercial products. Antisera to purified rat and human glutathione transferases were raised in rabbits by conventional procedures and were available in the laboratory. Antiserum against rat liver microsomal glutathione transferase was kindly provided by Dr. Ralf Morgenstern, Department of Biochemistry, University of Stockholm. Nitrocellulose filters were from Schleicher und Schuell, Dassel, Germany.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; BSP, bromosulphophthalein; IC_{50}, concn. of inhibitor that gives 50% inhibition.

Enzymes

Mouse mastocytoma cells (CXBGABMCT-1) were propagated intraperitoneally in CB_6F_1 mice. After 16 days of growth, the animals were killed and the cells harvested by rinsing the peritoneal cavity with phosphate-buffered saline (150 mM-NaCl/37 mM-KH_2PO_4/11 mM-Na_2HPO_4, pH 7.2). The cells were concentrated by centrifugation at 800 *g* for 5 min and resuspended in the same buffer. The suspension was sonicated for 6 × 5 s with a Branson Sonifier model S125. The disrupted cells were centrifuged at 10000 *g* for 15 min. The supernatant was further centrifuged at 105000 *g* for 60 min. The 105000 *g* sediment was resuspended and re-centrifuged, and the final sediment was resuspended in 50 mM-sodium phosphate buffer (pH 7.0)/1 mM-EDTA and used as the enzyme preparation representing leukotriene C synthase activity. Cytosolic glutathione transferases MII and MIII were isolated from the 105000 *g*-supernatant fraction of the mastocytoma cells by use of affinity chromatography on *S*-hexylglutathione–Sepharose and chromatofocusing as described previously [20]. Transferase MIII was further purified on CM-Sepharose. Mouse liver microsomal glutathione transferase was activated by treatment with *N*-ethylmaleimide and purified essentially by the method described for rat liver microsomal glutathione transferase [21].

Enzyme assays

The standard assay for determining enzyme activity with leukotriene A_4 methyl ester as substrate containing 25 mM-sodium phosphate buffer, pH 7.0, 0.5 mM-EDTA, 5 mM-glutathione and 12 μM-leukotriene A_4 methyl ester. The reaction was carried out at 30 °C for 1 min and was terminated by addition of 4 vol. of ethanol. The formation of leukotriene C_4 monomethyl ester was determined after ethyl acetate extraction as described previously [15]. The standard assay for determining activity with leukotriene A_4 contained 25 mM-sodium phosphate buffer, pH 7.0, 0.5 mM-EDTA, 5 mM-glutathione and 35 μM-leukotriene A_4. The reaction was carried out at 30 °C for 1 min and was terminated by addition of 1 vol. of methanol. The formation of leukotriene C_4 was determined after separation on reverse-phase h.p.l.c. as described previously [15]. Glutathione transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) was determined at 30 °C by using 1 mM-CDNB and 1 mM-glutathione in 0.1 M-sodium phosphate buffer, pH 6.5, and 1 mM-EDTA, as described previously [22]. Enzymic activities with 1,2-dichloro-4-nitrobenzene (DCNB), bromosulphophthalein (BSP) and ethacrynic acid were determined at 30 °C by published methods [22,23].

Inhibition studies

IC_{50} values, i.e. the concentration of inhibitor that gives 50% inhibition in the standard assay system, were determined at pH 6.5 with 1 mM-glutathione and 1 mM-CDNB as second substrate, and at pH 7.0 with 5 mM-glutathione and with 12 μM-leukotriene A_4 methyl ester as second substrate.

Isoelectric focusing

Isoelectric focusing was carried out in a 110 ml electrofocusing column (LKB), Pharmalyte pH 8–10.5 (Pharmacia) being used for generating the pH gradient. Isoelectric focusing was also carried out on polyacrylamide gels, containing 1% Triton X-100 and Pharmalyte pH 8–10.5.

Apparent subunit M_r

Apparent M_r values were determined on SDS/polyacrylamide gels [24].

Protein determination

Protein concentration was determined by the procedure of Peterson [25].

Immunoblotting

Electrophoretic transfer was performed from SDS/polyacrylamide gels on to nitrocellulose filters at 200 mA for 6 h [26]. The filters were then incubated with antisera against different rat and human glutathione transferases. The resulting immunocomplexes were detected by incubation with ^{125}I-labelled protein A, followed by autoradiography.

RESULTS

Leukotriene C_4 synthesis is known to be catalysed by membrane-bound as well as cytosolic enzymes. The total activity of leukotriene C_4 synthesis in mastocytoma cells, measured with leukotriene A_4 as substrate, was found to be distributed in a ratio of approx. 17:1 (7.8 versus 0.46 nmol/min per mg of protein) between the microsomal fraction (105000 *g* sediment) and the cytosol fraction (105000 *g* supernatant) obtained by differential centrifugation. The problem of identifying the enzyme(s) responsible for leukotriene C_4 biosynthesis in mouse mastocytoma cells was approached by parallel studies of the glutathione transferase activity, by using conventional substrates such as CDNB, and the leukotriene C_4-forming activity, with leukotriene A_4 or its methyl ester as substrate. In the present report we describe a membrane-bound enzyme responsible for the latter activity, which is distinct from earlier-known glutathione transferases. This enzyme will be referred to as leukotriene C synthase (leukotriene A_4:glutathione *S*-leukotrienyltransferase).

Purification and characterization of glutathione transferases from mouse mastocytoma cells

Essentially all glutathione transferase activity measured with CDNB in the cytosol fraction of mouse mastocytoma cells was adsorbed on the *S*-hexylglutathione affinity matrix. After elution of the bound transferases, two distinct forms were separated by chromatofocusing on a Mono P column (Fig. 1). The first peak of activity contained a single protein, whereas the transferase in the second peak required further purification on a CM-Sepharose column to attain homogeneity. On the basis of specific activities with several substrates, sensitivities to inhibitors, relative subunit M_r values, isoelectric points, and reactions with antibodies, the enzymes in the first and second peaks were identified as mouse glutathione transferases MII and MIII respectively, previously purified from mouse liver cytosol [20].

In addition to the isolation of cytosolic enzymes from mastocytoma cells, a membrane-bound glutathione transferase was purified from the microsomal fraction of mouse liver. Antibodies raised against the corresponding

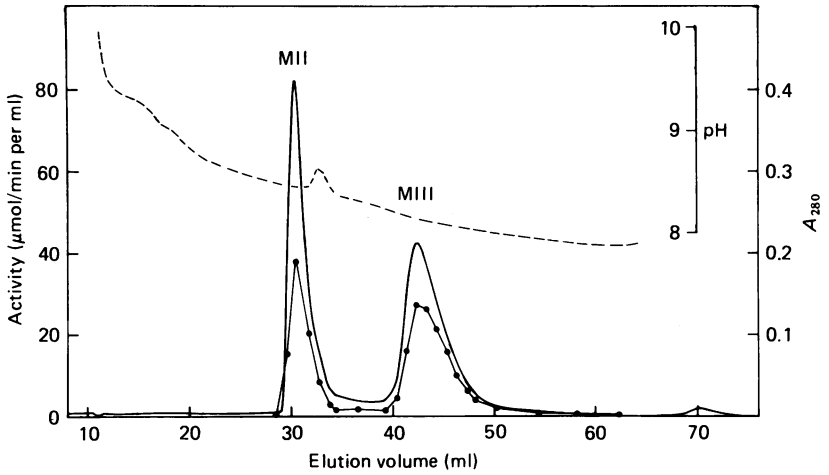

Fig. 1. Separation by chromatofocusing of cytosolic glutathione transferases from mouse mastocytoma cells

The cytosolic glutathione transferases were purified from mouse mastocytoma cells by affinity chromatography on *S*-hexylglutathione–Sepharose 6B. The pooled enzymes were then separated by chromatofocusing on a Mono P column (Pharmacia) as described in the Materials and methods section. Profiles are shown of activity with CDNB (●) and of pH (----) and A_{280} (——).

Table 1. Specific activities of enzymes catalysing the conjugation of glutathione with different electrophilic substrates

Abbreviations: LCS, leukotriene C synthase; MGT, microsomal glutathione transferase; LTA_4, leukotriene A_4.

	Specific activity (μmol/min per mg of protein)			
		Homogeneous glutathione transferases		
Substrate	Crude LCS	MGT	MII	MIII
LTA_4	0.008	< 0.0001	0.01	0.002
LTA_4 methyl ester	0.0001	< 0.0001	0.01	0.002
CDNB	< 0.04	42	102	229
DCNB	< 0.04	0.10	0.62	7.0
BSP	< 0.01	< 0.01	0.01	0.8
Ethacrynic acid	< 0.01	< 0.01	4.1	0.8

microsomal glutathione transferase in rat liver [21] cross-reacted with the mouse enzyme [27]. By using these antibodies, the presence of the same enzyme in the mastocytoma-cell microsomal fraction was clearly demonstrated by SDS/polyacrylamide-gel electrophoresis followed by immunoblot analysis. From this analysis, it was also evident that the concentration of the microsomal glutathione transferase was at least 10 times lower in mastocytoma cells than in mouse liver.

Specific activities

Specific activities for the crude leukotriene C synthase from mouse mastocytoma cells, the homogeneous cytosolic glutathione transferases MII and MIII and the purified hepatic microsomal glutathione transferase are given in Table 1. The specific activity of leukotriene C synthase was measured in a crude microsomal fraction, and is therefore not directly comparable with the values for the purified enzymes. Transferases MII and MIII from mouse mastocytoma cells catalysed a large number of reactions involving glutathione. They also effected the conjugation of leukotriene A_4 with glutathione. The specific activity for this reaction was 10 nmol/min per mg for MII and 2 nmol/min per mg for MIII. Microsomal glutathione transferase showed activity with CDNB and with DCNB, but did not, to any measurable extent, catalyse the conversion of leukotriene A_4 into leukotriene C_4 or of leukotriene A_4 methyl ester into leukotriene C_4 monomethyl ester. The experiments reported in Tables 1 and 2 were obtained with *N*-ethylmaleimide-activated microsomal glutathione transferase (cf. [21]); the unactivated enzyme was also inactive with leukotriene A_4 and the methyl ester. Leukotriene C synthase, as defined by the activity associated with the microsomal fraction of mastocytoma cells, catalysed the conjugation of leukotriene A_4 and leukotriene A_4 methyl ester with glutathione, but showed no measurable activity with any of the other substrates that were tested. For this enzyme leukotriene A_4 was the preferred substrate in comparison with the methyl ester, in contrast with the higher activity with the methyl ester displayed by cytosolic glutathione transferases [15,17].

Table 2. Sensitivities of microsomal and cytosolic glutathione transferases to different inhibitors, measured with CDNB as electrophilic substrate

The IC_{50} values for the different inhibitors were determined at pH 6.5, 30 °C with 1 mM-CDNB and 1 mM-glutathione as substrates. Abbreviations: MGT, microsomal glutatione transferase; n.d., not determined.

Inhibitor	IC_{50} (μM)		
	MGT	MII	MIII
S-BSP-glutathione	> 100	n.d.	n.d.
S-Hexyl-glutathione	> 100	27	18
Indomethacin	3	> 200	140
Tributyltin acetate	1.5	3.2	0.09
Triphenyltin chloride	0.5	6.3	0.05
BSP	15	66	6.6
Rose Bengal	0.15	7.9	1.1
Cibacron Blue	1	0.1	1.2

Table 3. Sensitivities of leukotriene C synthase and cytosolic glutathione transferases to different inhibitors with leukotriene A_4 methyl ester as electrophilic substrate

The IC_{50} values for the different inhibitors were determined at pH 7.0, 30 °C with 12 μM-leukotriene A_4 methyl ester and 5 mM-glutathione as substrates. Abbreviations: LCS, leukotriene C synthase.

Inhibitor	IC_{50} (μM)		
	LCS	MII	MIII
Indomethacin	1000	3000	400
Triphenyltin chloride	> 100	> 100	10
Rose Bengal	50	500	> 1000

Inhibition of activity with CDNB as substrate

IC_{50} values for cytosolic and microsomal glutathione transferases were determined with CDNB as the electrophilic second substrate (Table 2). Leukotriene C synthase was not active with CDNB, and was consequently not included in this series of experiments. Each of the enzymes was strongly inhibited ($IC_{50} \leqslant 0.1$ μM) by at least one of the inhibitors: microsomal glutathione transferase by Rose Bengal, transferase MII by Cibacron Blue, and transferase MIII by triphenyltin chloride and tributyltin acetate.

Inhibition of activity with leukotriene A_4 methyl ester as substrate

IC_{50} values were determined in the standard assay system for leukotriene A_4 methyl ester (Table 3). Leukotriene C synthase was inhibited by Rose Bengal, with an IC_{50} value of 50 μM. Triphenyltin chloride did not affect leukotriene C synthase at concentrations up to 100 μM. Indomethacin gave an IC_{50} value of 1 mM. Transferase MII was not strongly affected by any of the compounds tested. Transferase MIII was inhibited most effectively by triphenyltin chloride ($IC_{50} = 10$ μM).

Physical and immunochemical parameters

Apparent subunit M_r values were determined by SDS/polyacrylamide-gel electrophoresis. The microsomal glutathione transferase had an apparent subunit M_r of 17000. The cytosolic transferases MII and MIII had apparent subunit M_r values of 25 kDa and 27 kDa respectively. The isoelectric points of transferases MII and MIII were determined in an isoelectrofocusing column (LKB), by using a pH gradient between pH 8 and 10.5. Transferases MII and MIII had their isoelectric points at pH 8.7 and pH 8.5 respectively. The isoelectric point of the microsomal glutathione transferase was determined in a polyacrylamide gel that included 1% (w/v) Triton X-100, with a pH gradient between pH 8 and 10.5. The microsomal transferase had its isoelectric point at pH 8.8. The isoelectric point of leukotriene C synthase was estimated by solubilizing the enzyme from mouse mastocytoma-cell microsomal fraction with 1% Triton X-100, and focusing the solubilized material in a column, by using a pH gradient between pH 3.5 and 10; 1% Triton X-100 was included in the medium. The activity recovered gave a broad peak, the isoelectric point of which was estimated as approx. pH 6. Immunoprecipitation with antisera raised against different glutathione transferases showed close relationships between cytosolic glutathione transferase MIII and rat liver glutathione transferases 3-3 and 4-4, between MII and human placental glutathione transferase π, and between microsomal glutathione transferases originating from mouse and rat liver. The crude leukotriene C synthase could not be tested in an unambiguous manner, since the microsomal fraction is known to contain significant amounts of the cytosolic glutathione transferases [28].

DISCUSSION

Previous reports have shown that leukotriene A_4 is an intermediate in leukotriene C_4 biosynthesis [6] that is enzymically converted into leukotriene C_4 by conjugation with glutathione [7]. Different groups of investigators have reported that cytosolic glutathione transferases catalyse the formation of leukotriene C_4 from leukotriene A_4 and glutathione [15,16], as well as the formation of the monomethyl ester of leukotriene C_4 from leukotriene A_4 methyl ester [17]. The class Mu enzymes (i.e. human transferase μ and rat transferase 4-4) were the most efficient transferases in catalysing leukotriene C_4 synthesis. This class of cytosolic glutathione transferases is especially active with different epoxides as electrophilic substrates [29].

The biosynthesis of leukotriene C_4 is particularly prominent in certain cell types, e.g. basophilic leukaemia cells, mouse mastocytoma cells and eosinophilic leucocytes [30]. In the first two types of cells, the leukotriene C_4-forming activity has been localized to the membrane fraction [8,13,14]. A 10-fold-purified enzyme from rat basophilic leukaemia cells, catalysing the synthesis of leukotriene C_4, has properties that are different from those of microsomal glutathione transferase [14]. However, the nature of the enzyme(s) involved in leukotriene C_4 formation is still incompletely understood.

In rat liver the leukotriene C_4-synthesizing activity has been reported to be present in the 100000 *g* pellet [31], and a microsomal glutathione transferase in this tissue

has been described [21]. Cytosolic glutathione transferases that are membrane-associated have also been detected in liver microsomal fractions [28]. Thus the question remains whether leukotriene C_4 synthesis is catalysed by microsomal glutathione transferase, by cytosolic glutathione transferases, or by an enzyme separate from the other types of transferase, namely leukotriene C synthase.

Mouse mastocytoma cells have high capacity to form leukotriene C_4, and are therefore suitable for investigation of the enzymes involved in leukotriene C_4 formation. In the present investigation, two cytosolic glutathione transferases were purified and characterized from these cells. The isolated enzymes were found to be identical with transferases MII and MIII, previously described in mouse liver [20]. Further, a microsomal glutathione transferase was purified from mouse liver. The determination of substrate specificities showed that microsomal glutathione transferase had no activity with leukotriene A_4 or with leukotriene A_4 methyl ester as electrophilic substrates. This result indicates that the microsomal glutathione transferase is not responsible for leukotriene C_4 biosynthesis. The cytosolic glutathione transferases were able to catalyse the conversion of leukotriene A_4 into leukotriene C_4. However, the concentration of these enzymes in mastocytoma cells was rather low (0.2% of the total cytosolic protein concentration) as compared with the concentration in liver (2–5%), and the specific activities for these enzymes were consequently too low to account for leukotriene C_4 formation.

The microsomal and the cytosolic glutathione transferases could be distinguished by their differential sensitivities to inhibitors of the activity with CDNB. Inhibition experiments with leukotriene A_4 methyl ester as second substrate revealed a large difference in the sensitivity to Rose Bengal between leukotriene C synthase ($IC_{50} = 50\ \mu M$) and cytosolic glutathione transferases (IC_{50} values of 500 and $> 1000\ \mu M$). The IC_{50} values for transferases MII and MIII with leukotriene A_4 methyl ester as electrophilic substrate (Table 3) are higher than those obtained with CDNB (Table 2). This difference is, at least in part, explained by different degrees of saturation of the enzymes when acting on the alternative substrates. Leukotriene C synthases was less sensitive than transferase MIII to indomethacin and triphenyltin chloride. The isoelectric point of leukotriene C synthase differed from those of the microsomal and cytosolic transferases. Leukotriene C synthase was neutral or weakly acidic ($pI \approx 6$), whereas the cytosolic transferases and the microsomal transferase were all basic proteins ($pI \geqslant 8.5$).

In this investigation we have shown that a microsomal glutathione transferase, previously found in liver, is present in mouse mastocytoma cells, and that these cells also contained two forms of cytosolic glutathione transferase, which by all criteria used appeared identical with transferases MII and MIII of mouse liver. However, none of these enzymes was responsible for a quantitatively significant fraction of the leukotriene C_4 biosynthesis of mouse mastocytoma cells; the microsomal glutathione transferase had no activity with leukotriene A_4 as substrate, and the specific activity and concentration of the cytosolic glutathione transferases with leukotriene A_4 as electrophilic substrate were too low to account for leukotriene C_4 biosynthesis in the cells. Thus it is concluded that leukotriene C_4 formation in mouse mastocytoma cells is catalysed by a membrane-bound enzyme, leukotriene C synthase, distinct from cytosolic and microsomal glutathione transferases.

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