Activation and inhibition of microsomal glutathione transferase from mouse liver

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Mouse liver microsomal glutathione transferase was purified in an *N*-ethylmaleimide-activated as well as an unactivated form. The enzyme had a molecular mass of 17 kDa and a pI of 8.8. It showed cross-reactivity with antibodies raised against rat liver microsomal glutathione transferase, but not with any of the available antisera raised against cytosolic glutathione transferases. The fully *N*-ethylmaleimide-activated enzyme could be further activated 1.5-fold by inclusion of 1 μ M-bromosulphophthalein in the assay system. The latter effect was reversible, which was not the case for the *N*-ethylmaleimide activation. At 20 μ M-bromosulphophthalein the activated microsomal glutathione transferase was strongly inhibited, while the unactivated form was activated 2.5-fold. Inhibitors of the microsomal glutathione transferase from mouse liver showed either about the same I_{50} values for the activated and the unactivated form of the enzyme, or significantly lower I_{50} values for the activated form compared with the unactivated form. The low I_{50} values and the steep slope of the activity-versus-inhibitor-concentration curves for the latter group of inhibitors tested on the activated enzyme indicate a co-operative effect involving conversion of activated enzyme into the unactivated form, as well as conventional inhibition of the enzyme.

INTRODUCTION

Glutathione transferases are a group of enzymes that catalyse the conjugation of various electrophiles with glutathione (Jakoby, 1978). The main function of the enzymes is believed to be detoxication of reactive intermediates in the metabolism of xenobiotics and of compounds endogenous to the cell. The reaction products are water-soluble conjugates that are excreted in urine and bile (Jakoby & Habig, 1980; Mannervik, 1985). The glutathione transferases occur in multiple forms, and the cytosolic mammalian enzymes can be divided into three distinct classes, Alpha, Mu and Pi (Mannervik *et al.*, 1985). In addition, a microsomal form of glutathione transferase has been isolated and characterized from rat liver and has been shown to possess characteristics that are clearly distinct from those of the cytosolic transferases such as amino acid sequence, subunit M_r , enzymic properties and ability to be activated by thiol reagents (Morgenstern *et al.*, 1979, 1980, 1982, 1985).

Our present studies concern the biosynthesis of leukotriene C_4 in mouse mastocytoma cells, which involves conjugation of leukotriene A_4 with the thiol group of glutathione. It has been shown that this reaction, in rat basophilic leukaemia cells, is catalysed by a membrane-bound enzyme (Jakschik *et al.*, 1982; Bach *et al.*, 1984; Yoshimoto *et al.*, 1985). For this reason it was important to characterize the mouse microsomal glutathione transferase and to determine its properties, such as substrate specificity, sensitivity to inhibitors and physico-chemical characteristics, in order to be able to evaluate its possible contribution to leukotriene C_4 synthesis.

MATERIALS AND METHODS

Materials

Hydroxyapatite was from Bio-Rad (Richmond, CA, U.S.A.); Sephadex G-25, CM-Sepharose, Pharmalyte

pH 8–10.5 and an isoelectric-focusing kit were from Pharmacia (Uppsala, Sweden); nitrocellulose membrane filters were from Schleicher und Schuell (Dassel, Germany); Triton X-100, NEM and glutathione were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); rat liver microsomal glutathione transferase and rabbit antiserum directed against this enzyme were kindly provided by Dr. R. Morgenstern of this Department; *S*-hexylglutathione and *S*-*p*-bromobenzylglutathione were synthesized by the method of Vince *et al.* (1971). All other chemicals were of highest purity and were purchased from common commercial sources.

Assay of enzyme activity

Enzyme activity was measured spectrophotometrically at 340 nm in a reaction system (30 °C) containing 5 mM-glutathione, 1 mM-CDNB, 5% (v/v) ethanol (solvent for CDNB), 1 mM-EDTA and 0.1 M-sodium phosphate buffer, pH 6.5. A unit of enzyme activity catalyses the formation of 1 μ mol of *S*-2,4-dinitrophenylglutathione/min. Specific activities with DCNB, cumene hydroperoxide, 4-hydroxyalkenals, leukotriene A_4 and leukotriene A_4 methyl ester were determined by published methods (Booth *et al.*, 1961; Lawrence & Burk, 1976; Alin *et al.*, 1985; Söderström *et al.*, 1985).

Protein determination

Protein concentration was determined by the procedure of Peterson (1977).

Purification of the enzyme

Liver microsomes (microsomal fraction) were prepared from male mice (strain CB₆F₁; 25 g body wt.). The microsomal glutathione transferase was purified in the unactivated form essentially by the method for the NEM-activated rat liver enzyme as described by Morgenstern *et al.* (1982). The purification procedure was

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; NEM, *N*-ethylmaleimide; BSP, bromosulphophthalein; I_{50} , concentration of inhibitor causing half-maximal inhibition.

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modified by an additional hydroxyapatite chromatography after the CM- Sephadex step.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed with 15% (w/v) acrylamide and 0.4% (w/v) NN' -methylenebisacrylamide in 1 mm-thick slab gels as described by Laemmli (1970).

Immunoblotting

Electrophoretic transfer (200 mA, 4 h) of mouse liver microsomal glutathione transferase was performed from SDS/polyacrylamide gels (1.5 mm-thick slab gel; 15% polyacrylamide) on to a nitrocellulose filter as described by Berzins *et al.* (1983). The nitrocellulose filter was then incubated with antiserum against rat liver microsomal glutathione transferase. The rat transferase was also transferred as a positive control. The immunocomplexes were revealed by reaction with ^{125}I -labelled protein A and autoradiography.

Kinetic studies

Enzyme efficiency, expressed as k_{cat}/K_m , was measured in the standard assay system, except that the CDNB concentration was in the range 1–10 μM , providing first-order conditions with respect to this substrate (Danielson & Mannervik, 1985). Inhibition studies were performed as described by Tahir *et al.* (1985).

Determination of pI

pI was determined on polyacrylamide gel by using Pharmalyte pH 8–10.5 for creating the pH gradient. Triton X-100 (1%, w/v) was included in the gel in order to keep the enzyme from precipitating.

RESULTS AND DISCUSSION

Purification

Glutathione transferase was purified from mouse liver microsomes without prior activation (cf. Morgenstern & DePierre, 1983). The purification factor was 19 and the total recovery of the activity was 21%, corresponding to a total yield of 2.5 mg of protein from 14 g of mouse liver. The mouse microsomal glutathione transferase in activated form was purified by the procedure of Morgenstern *et al.* (1982).

Molecular properties of mouse liver microsomal glutathione transferase

When SDS/polyacrylamide-gel electrophoresis was performed with purified mouse and rat microsomal glutathione transferase loaded in the same or in different lanes, they co-migrated to the same position in the gel (Fig. 1). The molecular mass of the rat liver microsomal glutathione transferase has been determined as 17237 Da by complete amino acid sequence analysis (Morgenstern *et al.*, 1985). On the basis of similarities in the electrophoretic mobilities of the rat and the mouse enzymes, a molecular mass of approx. 17000 Da can be inferred for the microsomal glutathione transferase from mouse liver.

The pI value of the mouse liver microsomal glutathione transferase was determined as 8.8 by gel isoelectric focusing. Parallel experiments with the microsomal rat enzyme confirmed the higher pI value of 10.1 previously

Fig. 1. Analysis of purified glutathione transferase from mouse liver microsomes by SDS/polyacrylamide gel electrophoresis

(a) Immunoblot analysis of purified microsomal transferase electroblotted on to nitrocellulose paper (Berzins *et al.*, 1983). All lanes contained the mouse enzyme, except the second lane from the left, which contained the rat microsomal enzyme for comparison. The seven lanes (from left to right) were developed with antisera raised against the following rat glutathione transferases: microsomal (lanes 1 and 2), 1-1 (3), 2-2 (4), 3-3 (5), 4-4 (6) and 7-7 (7). The immunocomplexes were revealed by reaction with ^{125}I -labelled Protein A and autoradiography. (b) Detection of protein by Coomassie Brilliant Blue staining. Purified glutathione transferase from mouse liver microsomes (left) and M_r markers (right).

reported for that enzyme (Morgenstern & DePierre, 1983).

In immunoblotting experiments the mouse microsomal glutathione transferase reacted strongly with antibodies against the rat microsomal glutathione transferase (Fig. 1). The mouse enzyme did not show any cross-reactivity in immunoblot assays with any of the antibodies raised against the different classes of cytosolic glutathione transferases (cf. Mannervik *et al.*, 1985).

Activation

The purified enzyme was incubated on ice with different NEM concentrations for different periods of time (Fig. 2). The enzyme was fully activated after incubation with 10 mM-NEM for 8–10 min. Higher concentrations of NEM did not result in further activation of the enzyme (results not shown). The activation was terminated by addition of 10 mM-glutathione and resulted in an 8.5-fold activation. NEM is thought to attach to the SH group of a cysteine residue in the protein (Morgenstern & DePierre, 1983). The activating effect was not lost upon dilution, which indicates a covalent modification of the enzyme, resulting in an enhancement of the enzyme activity with CDNB.

The NEM-activated enzyme could be further activated (1.5-fold) by including 1 μM -BSP in the assay system (Fig. 3). This effect was reversible, as demonstrated by

Fig. 2. Activation of purified glutathione transferase from mouse liver microsomes by NEM

Enzyme was incubated at 0 °C with 1 mM (●), 5 mM (△) and 10 mM (○)-NEM; portions were taken for determination of transferase activity in the standard assay system at the points indicated.

Fig. 3. Effect of BSP on purified glutathione transferase from mouse liver microsomes

Enzyme maximally activated with 10 mM-NEM (●) or unactivated (○) was assayed with the concentrations of bromosulphophthalein indicated. No time-dependence of the effect of BSP was found (cf. Fig. 2).

dilution. The unactivated enzyme was also activated by BSP: about 2.5-fold at 20 μM (Fig. 3). At higher concentrations BSP acted as an inhibitor. BSP has been found to bind at more than one site per subunit of cytosolic glutathione transferase 3-3 (Jakobson *et al.*, 1979); at low concentrations it binds non-co-operatively, but at higher concentrations additional weaker non-specific binding comes into play. A similar behaviour may explain the results obtained with the microsomal glutathione transferase. The effect of BSP on the unactivated enzyme may be related to the conformational change believed to occur upon NEM activation. However, the additional activation of NEM-treated enzyme

Table 1. Specific activities of microsomal glutathione transferase from mouse liver

Abbreviations: n.d., not detectable in the assay system; LTA₄, leukotriene A₄.

Substrate	Enzyme...	Activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	
		Activated	Unactivated
CDNB		42	5.7
DCNB		0.10	0.16
BSP		< 0.01	< 0.01
Ethacrynic acid		< 0.01	< 0.01
4-Hydroxynonenal		n.d.	n.d.
LTA ₄		< 0.0001	< 0.0001
LTA ₄ methyl ester		< 0.0001	< 0.0001
Cumene hydroperoxide		1.9	1.9

Table 2. Kinetic constants for microsomal glutathione transferase from mouse liver

The parameters were determined with variable CDBN concentration at 5 mM-glutathione.

Constant	Mouse liver microsomal glutathione transferase...	Activated	Unactivated
$k_{\text{cat.}}$ (s^{-1})		10	2
K_{m} (μM)		45	30
$k_{\text{cat.}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)*		0.16	0.11
$k_{\text{cat.}}/K_{\text{m}}$ ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)†		0.22	0.07

* Determined at low CDBN concentration, as described in the Materials and methods section.

† Calculated from the above values of K_{m} and $k_{\text{cat.}}$.

afforded by BSP shows that the effects of the two activators are not identical and interchangeable. Other alkylating compounds tested as potential activators of the purified microsomal glutathione transferase from mouse liver were 4-hydroxypentenol, 4-hydroxynonenol, 4-hydroxydodecenol (all at 100 μM) and ethacrynic acid (1 and 5 mM). Further, iodoacetamide (10 mM), cystamine (10 mM) and CDBN (1 and 10 mM) were tested directly on the crude enzyme in mouse liver microsomes. However, none of these compounds gave any measurable activation. Thus the activation is dependent on effects other than the mere blocking of an SH group.

Kinetic properties

The specific activities with several standard substrates for the glutathione transferases were low for the mouse microsomal enzyme, except with CDBN (Table 1). Only with the latter substrate was the activation by NEM noticeable. The pH-dependence of the enzyme activity with CDBN showed a broad peak, with a maximum at about pH 7. Essentially the same results were previously obtained with the enzyme from rat microsomes (Morgens-tern & DePierre, 1983). Of particular interest was the lack of activity with the products of arachidonic acid

Table 3. Inhibition of activated and unactivated microsomal glutathione transferase from mouse liver

Abbreviation: n.t., not tested.

Inhibitor	Enzyme...	I_{50} value (μM)	
		Activated	Unactivated
Indomethacin		3	5
Tributyltin acetate		1.5	10
Triphenyltin chloride		0.5	1
Bromosulphophthalein		15	100
Rose Bengal		0.15	13
Cibacron Blue F3G-A		1	25
S-Hexylglutathione		> 100	> 100
S-p-Bromobenzylglutathione		> 100	n.t.

metabolism, namely leukotriene A_4 and 4-hydroxy-nonanal.

Constants for the steady-state kinetics of microsomal glutathione transferase in activated and unactivated form are shown in Table 2. The $k_{\text{cat.}}/K_m$ value, which is a measure of the catalytic efficiency (Fersht, 1985), was determined at low CDNB concentration (1–10 μM), at which $k_{\text{cat.}}/K_m$ is an apparent second-order rate constant (Danielson & Mannervik, 1985), and was also calculated from the values of $k_{\text{cat.}}$ and K_m given in Table 2. Both estimations showed that the $k_{\text{cat.}}/K_m$ value increased approx. 2-fold after activation of the enzyme with NEM. The values obtained were similar to the $k_{\text{cat.}}/K_m$ value determined for the rat enzyme ($0.10 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$; Morgenstern & DePierre, 1983).

Inhibition

The inhibitors tested on mouse liver microsomal glutathione transferase (Table 3) could be divided basically into three groups, characterized by: (1) similar I_{50} values for the activated and the unactivated form of the enzyme; (2) significantly higher I_{50} values for the unactivated enzyme as compared with the activated enzyme; and (3) activation of the enzyme at low concentrations and inhibition at higher concentrations. S-Hexylglutathione and S-p-bromobenzylglutathione were weak inhibitors and will not be discussed further.

Indomethacin, triphenyltin chloride and tributyltin acetate show approximately the same I_{50} value for the activated and the unactivated form of the enzyme (Table 3). The maximum slope of the inhibition curves for the activated enzyme is approx. -58 at 50% inhibition, characteristic for linear inhibition of monomeric or non-co-operative oligomeric enzymes (Fig. 4) (Tahir & Mannervik, 1986). In contrast, Rose Bengal and Cibacron Blue show significantly different I_{50} values for the activated and the unactivated form of the enzyme. The inhibition curve for the activated form shows a maximal negative slope significantly higher than 58, indicating a co-operative effect (Fig. 5). Apparently Rose Bengal and Cibacron Blue cause reversal of the conformational change induced by NEM and, thereby, elimination of the activation.

BSP was the only compound tested that both activated and inhibited the enzyme (Fig. 3). Inhibition was observed at high concentrations, which suggests binding

Fig. 4. Inhibition of purified glutathione transferase from mouse liver microsomes by tributyltin acetate (O) and indomethacin (Δ)

The shape of the curves is typical of simple linear inhibition (cf. Tahir & Mannervik, 1986). The experiments were carried out with NEM-activated enzyme, but the effects were not significantly different with unactivated enzyme.

Fig. 5. Inhibition of purified glutathione transferase from mouse liver microsomes by Rose Bengal (O, ●) and Cibacron Blue (Δ, ▲)

The steep slopes of the curves (cf. Fig. 4) demonstrate a co-operative inhibition. The open symbols (O, Δ) represent enzyme maximally activated by preincubation with NEM, and the closed symbols (●, ▲) represent unactivated enzyme.

of BSP to a second site, resulting in elimination of the activating effect at low concentrations (Fig. 3). The activated enzyme was inhibited non-competitively, with respect to CDNB, by BSP ($K_{ii} = 4 \mu\text{M}$ and $K_{is} = 2.5 \mu\text{M}$; inhibition constants defined by Cleland, 1963).

Thus the compounds in the first group act as normal linear inhibitors that are equally strong with activated and unactivated glutathione transferase. The second group of compounds exerts two linked effects, i.e. normal inhibition of enzyme activity accompanied and augmented by conversion of the activated enzyme into the unactivated form. Apparently BSP acts as an activator as well as an inhibitor of the latter category. The results presented consequently lend strong support to the previous proposal of a conformational change as an integral part of the activation mechanism.

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