Purification and characterization of a new cytosolic glutathione *S*-transferase (glutathione *S*-transferase X) from rat liver

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A hitherto unknown cytosolic glutathione *S*-transferase from rat liver was discovered and a method developed for its purification to apparent homogeneity. This enzyme had several properties that distinguished it from other glutathione *S*-transferases, and it was named glutathione *S*-transferase X. The purification procedure involved DEAE-cellulose chromatography, $(\text{NH}_4)_2\text{SO}_4$ precipitation, affinity chromatography on Sepharose 4B to which glutathione was coupled and CM-cellulose chromatography, and allowed the isolation of glutathione *S*-transferases X, A, B and C in relatively large quantities suitable for the investigation of the toxicological role of these enzymes. Like glutathione *S*-transferase M, but unlike glutathione *S*-transferases AA, A, B, C, D and E, glutathione *S*-transferase X was retained on DEAE-cellulose. The end product, which was purified from rat liver 20000 *g* supernatant about 50-fold, as determined with 1-chloro-2,4-dinitrobenzene as substrate and about 90-fold with the 1,2-dichloro-4-nitrobenzene as substrate, was judged to be homogeneous by several criteria, including sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, isoelectric focusing and immunoelectrophoresis. Results from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration indicated that transferase X was a dimer with M_r about 45 000 composed of subunits with M_r 23 500. The isoelectric point of glutathione *S*-transferase X was 6.9, which is different from those of most of the other glutathione *S*-transferases (AA, A, B and C). The amino acid composition of transferase X was similar to that of transferase C. Immunoelectrophoresis of glutathione *S*-transferases A, C and X and precipitation of various combinations of these antigens by antisera raised against glutathione *S*-transferase X or C revealed that the glutathione *S*-transferases A, C and X have different electrophoretic mobilities, and indicated that transferase X is immunologically similar to transferase C, less similar to transferase A and not cross-reactive to transferases B and E. In contrast with transferases B and AA, glutathione *S*-transferase X did not bind cholic acid, which, together with the determination of the M_r , shows that it does not possess subunits Ya or Yc. Glutathione *S*-transferase X did not catalyse the reaction of menaphthyl sulphate with glutathione, and was in this respect dissimilar to glutathione *S*-transferase M; however, it conjugated 1,2-dichloro-4-nitrobenzene very rapidly, in contrast with transferases AA, B, D and E, which were nearly inactive towards that substrate. Thus glutathione *S*-transferase X has properties that differ from those of the known glutathione *S*-transferases in several respects, although it is closely related to transferases A and C. However, reduction and structural investigations indicated that glutathione *S*-transferase X represents neither an oxidized or a reduced form nor a proteolytic product or precursor of either transferase A or C. This new form appears to be especially important, in that it was substantially more potent in inactivating a diol epoxide, the vicinal dihydrodiol non-bay-region epoxide *t*-10,11-epoxy-*r*-8,*t*-9-dihydroxy-8,9,10,11-tetrahydrobenz[*a*]anthracene, than any of the other major glutathione *S*-transferases.

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

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The glutathione *S*-transferases (EC 2.5.1.18) are a group of enzymes that catalyse the conjugation of a wide variety of compounds with GSH (Jakoby *et al.*, 1976a). Several transferases have been identified in the rat liver cytosol, microsomal and mitochondrial fractions (Habig *et al.*, 1974; Glatt & Oesch, 1977; Friedberg *et al.*, 1979; Kraus, 1980; Stasiecki *et al.*, 1980), in other organs, e.g. lung and kidney (Moron *et al.*, 1979; van Cantfort *et al.*, 1979), and in other species, including man (Simons & Vander Jagt, 1977; Golan *et al.*, 1980; Oesch *et al.*, 1980). The rat liver cytosol glutathione *S*-transferases are named transferases AA, A, B, C, D and E in the reverse order of their elution from a CM-cellulose column (Jakoby *et al.*, 1976a), and all of these except transferase D have been purified to homogeneity (Habig *et al.*, 1974, 1976). A seventh glutathione *S*-transferase has also been partially purified from rat liver cytosol by Gillham (1971), and was subsequently named glutathione *S*-transferase M in view of its reaction with menaphthyl sulphate. In addition to the intrinsic biochemical interest, information on the various glutathione *S*-transferase forms is of high practical importance in that they constitute one of the enzyme families that are involved in the control of reactive metabolites (Oesch, 1973; Jerina & Daly, 1974; Sims & Grover, 1974; Jakoby *et al.*, 1976a; Glatt *et al.*, 1981).

The rat liver cytosolic glutathione *S*-transferases that have been investigated are not immunologically cross-reactive, with the exception of transferases A and C, in contrast with the human liver enzymes, which all have similar immunological properties (Fleischner *et al.*, 1976). Unexpectedly, we found on immunoelectrophoresis of rat liver 100 000 g supernatant and antiserum raised against glutathione *S*-transferase C, three precipitation crescents. We were interested in having several immunologically closely related glutathione *S*-transferases in order to later elucidate the mechanism of their diversification and their differential role in the control of reactive metabolites. We have therefore purified and characterized the third transferase that is immunologically cross-reactive to transferase A and C.

Experimental

Materials

GSH, 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene and *p*-nitrobenzyl chloride (Sigma Chemical Co.), menaphthyl sulphate (Calbiochem), 1,2-epoxy-(*p*-nitrophenoxy)propane (Eastman), 2-(4-nitrophenyl)ethyl bromide (EGA-Chemie), Ultrodex gel and Ampholines (LKB Produkter), DE-52 DEAE-cellulose and CM-52 CM-cellulose (Whatman Biochemicals), Sephadex G-100 and Sepharose

4B (Pharmacia) were obtained from the commercial sources indicated.

Enzyme assays

The glutathione *S*-transferase assays were performed as described by Habig *et al.* (1974) at 25°C except for the assay with menaphthyl sulphate, which was performed at 37°C as described by Gillham (1971). One unit of activity is defined as the amount of enzyme catalysing the formation of 1 nmol of product/min under the specific assay conditions. 1-Chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were recrystallised twice from ethanol/water before use. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of GSH-Sepharose 4B

GSH was coupled to CNBr-activated Sepharose 4B by using the procedure described by Morrison *et al.* (1976), with some modifications. A solution of 10 g of ethylenediamine in 500 ml of tetrahydrofuran was rapidly added to a stirred solution of 20 g of maleic anhydride in 500 ml of tetrahydrofuran kept in an ice bath. The reaction mixture was kept cold for an additional 2 min and then stirred at room temperature for 4 h. The resulting precipitate was washed in a Buchner funnel with 1 litre of tetrahydrofuran. After washing, most tetrahydrofuran was removed by suction. The precipitate was transferred to 100 ml of 1 M-Na₂CO₃ solution and the pH was adjusted to 9.8 with solid Na₂CO₃. The solution was cooled to 4°C and added to 400 ml of CNBr-activated Sepharose 4B prepared as described by March *et al.* (1974). The suspension was stirred at 4°C for 20 h and then transferred to a Buchner funnel, where the gel was washed with 5 litres of 100 mM-sodium phosphate buffer, pH 7. The wet gel was then added to 50 ml of cold 0.5 M-Na₂CO₃ containing 25 g of GSH. The pH of this solution was adjusted with solid Na₂CO₃ to 7. After overnight stirring at 4°C, the gel was washed with 10 litres of 50 mM-sodium phosphate buffer, pH 8.4, containing 100 mM-KCl.

Purification of glutathione *S*-transferase X

Thirty male Sprague-Dawley rats (220–240 g; Versuchstieranstalt Wiga, Sulzfeld, Germany) were killed by a blow on the head. The livers were removed, washed with ice-cold distilled water and homogenized, and the homogenate was centrifuged at 20 000 g as described by Jakoby and co-workers as initial steps in the isolation of glutathione *S*-transferases AA, A, B and C (Habig *et al.*, 1974). The resulting supernatant was applied to a column (30 cm × 10 cm) of DEAE-cellulose that had been equilibrated with 10 M-Tris/HCl buffer, pH 8. Most of the glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene as substrate was eluted

as a single peak. This fraction (fraction 1) was retained for the purification of the transferase A, B and C. When no further transferase activity was eluted, the column was rinsed with 3 litres of 10mM-Tris/HCl buffer, pH8, containing 20mM-KCl. Finally, the bound activity was eluted with 10mM-Tris/HCl buffer, pH8, containing 100mM-KCl. The transferase activity was found in a single peak (fraction 2), active fractions were combined and protein was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ (660g/l). The precipitate was dissolved in a minimum volume of 50mM-sodium phosphate buffer, pH8.4, containing 100mM-KCl, dialysed for 18h against 2×5 litres of this buffer and applied to a GSH-Sepharose 4B column (30cm $\times$ 5 cm) that had been equilibrated with dialysis buffer. Most of the protein was eluted with this buffer. Bound transferase activity was eluted with 50mM-sodium phosphate buffer, pH7.3, containing 100mM-KCl and 5mM-GSH. Active fractions were combined, concentrated by ultrafiltrations to give a final volume of 5 ml, dialysed against 1×5 litres of 10mM-potassium phosphate buffer, pH6.7, and applied to a CM-cellulose column (20cm $\times$ 2.5cm) that had been equilibrated with the same buffer. Transferase activity was not retained on the column, and all active fractions were combined and concentrated by ultrafiltration. The concentrated protein solution was dialysed against 1 litre of 50mM-sodium phosphate buffer, pH7, containing 10% (v/v) glycerol and stored at -70°C .

Purification of glutathione S-transferases A, B and C

Transferases A, B and C were isolated from the rat livers used for the purification of transferase X. Transferase B was purified by the method of Habig *et al.* (1974). Initially, transferases A and C were also purified by this method. Later a modified procedure, which included chromatography on DEAE-cellulose (10mM-Tris/HCl buffer, pH8) and then on CM-cellulose (10mM-potassium phosphate buffer, pH6.7), was used. The transferases were eluted from the CM-cellulose column as described by Habig *et al.* (1974). Activity was monitored with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates. The second and fourth peaks of transferase activity were collected and concentrated by ultrafiltration to 20ml. The two protein solutions were each dialysed against 1×5 litres of 50mM-sodium phosphate buffer, pH8.4, containing 100mM-KCl and purified separately by affinity chromatography on GSH-Sepharose 4B, as described above. The transferase activity, which was eluted from the affinity column with GSH, was collected, concentrated by ultrafiltration, dialysed against 50mM-sodium phosphate buffer, pH7, containing 10% (v/v) glycerol and stored at -70°C .

Electrophoresis

SDS/polyacrylamide-gel electrophoresis in 2mm-thick slab gels containing 10% acrylamide was performed by using a modification of the system described by Davis (1964), in which SDS had been included in all buffer solutions. Gels were stained with Coomassie Blue.

Isoelectric focusing

Isoelectric focusing was performed at 4°C in Ultradex flat beds by using an LKB Multiphor apparatus as described by the manufacturers. A 5mg sample of glutathione transferase X was focused. The pH gradient was established by using ampholyte solutions in either the range 6.5–11 (test for the presence of other glutathione *S*-transferases) or the range 5–8 (determination of isoelectric point). Gels contained 10% (v/v) glycerol. After 16h, isoelectric focusing was complete, and the gel was fractionated by using the grid provided. Gel fractions were eluted with 2ml of distilled water, and the pH and enzyme activity towards 1-chloro-2,4-dinitrobenzene were measured. Analytical isoelectric focusing was performed by using the procedure described by the manufacturer (Pharmacia Fine Chemicals, 1982).

Antibody production

Each of three male New Zealand White rabbits (2–3 kg) received subcutaneous injections of a total of 200 μg of either transferases C, X or B in Freund's complete adjuvant at several sites. After 1 month the procedure was repeated. Then 1 month later the rabbits were each given a booster injection of another 200 μg of the appropriate transferase in buffered saline. Blood was taken from the ear vein 14 days later.

Immunoelectrophoresis

Immunoelectrophoresis was performed at 4°C at 5 V/cm in 1% agarose gels containing 25mM-barbital buffer, pH8.6. Gels were stained with Coomassie Blue.

Amino acid analysis

The purified protein was hydrolysed in 6M-HCl for 20h or 72h in evacuated sealed tubes. Norleucine was added before hydrolysis as an internal standard. The hydrolysed samples were analysed by using a Beckman amino acid analyser. Tryptophan was determined spectrophotometrically as described by Basha & Roberts (1977).

Test for cholic acid binding by glutathione S-transferases X and B

The binding of cholic acid to either transferase X or B was studied by an equilibrium chromato-

graphic method (Hayes *et al.*, 1980). A Bio-Gel A-0.5m column (34 cm $\times$ 2.5 cm) was equilibrated at 4°C with 20 mM-sodium phosphate buffer, pH 7.4, containing 100 mM-NaCl and [3 H]cholic acid (10 nM, 150 c.p.m./ml). The flow rate was 23 ml/h, and 2.7 ml fractions were collected. The elution volume of Blue Dextran was 71 ml and that of [3 H]cholic acid was 180 ml. Samples (1 ml) containing either 17 mg of partially purified transferase B (specific activity 5600 units/mg with 1-chloro-2,4-dinitrobenzene as substrate) or 10 mg of apparently homogeneous transferase X were dialysed for 18 h against 500 ml of the equilibration buffer and applied to the column. Radioactivity, A_{280} and transferase activity towards 1-chloro-2,4-dinitrobenzene were monitored in the eluate.

Results

Enzyme purification

The results of a typical purification of transferase X are summarized in Table 1. The starting material for the preparation was rat liver 20000 g supernatant. The enzyme was also purified from rat liver 100000 g supernatant with similar results. DEAE-cellulose chromatography was chosen as the initial step, because it was also suitable for the purification of transferases A, B and C, which were not retained by this column, whereas transferase X was bound at low ionic strength (10 mM-Tris/HCl buffer, pH 8). The loss of transferases A, B and C is most probably responsible for the decrease in activity towards 1-chloro-2,4-dinitrobenzene observed in the subsequent eluate (fraction 2) obtained at higher ionic strength (100 mM-KCl in the same buffer). The latter eluate (fraction 2) was enriched 3.5-fold in activity towards menaphthyl sulphate. $(\text{NH}_4)_2\text{SO}_4$ precipitation was used to concentrate the eluate.

Affinity chromatography of the concentrated protein solution on GSH-Sephrose 4B and elution of retained protein with GSH led to a total loss of

Fig. 1. SDS/polyacrylamide-gel electrophoresis of glutathione *S*-transferase X and B

Glutathione *S*-transferase B purified by the method of Habig *et al.* (1974) and glutathione *S*-transferase X from the final purification step described in the present paper were applied to the gel. From left to right: 20 µg of glutathione *S*-transferase X, 2 µg of glutathione *S*-transferase X, 30 µg of glutathione *S*-transferase B and 5 µg of glutathione *S*-transferase B. The two bands of transferase B correspond to its non-identical subunits (Hayes *et al.*, 1980).

Table 1. Purification to apparent homogeneity of rat liver cytosolic glutathione *S*-transferase X

One unit of activity is defined as the amount of enzyme catalysing the formation of 1 nmol of product/min under the specific assay conditions. Numbers in parentheses give the percentage of total yield for total units, or the purification factor for specific activity. A, Substrate: 1-chloro-2,4-dinitrobenzene; B, 1,2-dichloro-4-nitrobenzene; C, menaphthyl sulphate. Abbreviation: N.D., not detected.

Purification step	Substrate ...	10 ⁻⁶ $\times$ Total activity (units)			Specific activity (units/mg of protein)		
		A	B	C	A	B	C
2 $\times$ 10 ⁴ g supernatant		14.1 (100)	1.01 (100)	0.0736 (100)	435 (1.0)	31.2 (1.0)	2.3 (1.0)
DEAE-cellulose chromatography		0.69 (4.90)	0.167 (16.5)	0.0350 (47.5)	158 (0.4)	38.2 (1.2)	8.0 (3.5)
(NH ₄) ₂ SO ₄ precipitation		0.75 (5.32)	0.210 (19.9)	0.0310 (42.1)	168 (0.4)	45.4 (1.5)	7.1 (3.1)
GSH-Sephrose 4B chromatography		0.49 (3.47)	0.044 (4.36)	N.D.	11 100 (25.5)	1010 (32.4)	N.D.
CM-cellulose chromatography		0.32 (2.27)	0.040 (3.96)	N.D.	21 500 (49.4)	2710 (87.0)	N.D.

activity towards menaphthyl sulphate. This activity was found in the protein fractions not retained on the affinity column. Most glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene (65% of the applied activity) and some of the activity towards 1,2-dichloro-4-nitrobenzene (22% of the applied activity) was then eluted by GSH from the affinity column. By this step the enzyme preparation was highly enriched in activity towards 1-chloro-2,4-dinitrobenzene (66-fold) and 1,2-dichloro-4-nitrobenzene (22-fold). However, the protein solution was slightly red, and SDS/polyacrylamide-gel electrophoresis showed two protein-staining bands, with M_r about 23 000 and M_r less than 10 000. The contaminating protein was retained by the CM-cellulose, whereas the transferase did not bind. With respect to the 20 000 g supernatant fraction of the liver homogenate, the end product was purified about 50-fold as determined with 1-chloro-2,4-dinitrobenzene as substrate and about 90-fold as determined with 1,2-dichloro-4-nitrobenzene as substrate.

Purity of the enzyme preparation

SDS/polyacrylamide-gel electrophoresis of the final preparation showed a single band even when large amounts of protein were applied to the gel (Fig. 1). Flat-bed isoelectric focusing of 5 mg of transferase X in a pH gradient from 6.5–11 (results not

shown) resulted in a single band of activity towards 1-chloro-2,4-dinitrobenzene that focused at pH 7, indicating that the transferase X was not contaminated by known glutathione transferases with different isoelectric points (AA, A, B and C). Antiserum raised against the transferase X preparation in New Zealand White rabbits gave a single precipitation band when tested in a double-diffusion assay (results not shown) or a single unsplit crescent after immunoelectrophoresis (Fig. 2b) with purified transferase X as the antigen.

Properties of the enzyme

The M_r was estimated in the presence of standard proteins by gel filtration (Fig. 3a) and by SDS/polyacrylamide-gel electrophoresis (Fig. 3b). The first method gave an M_r value of 45 000, whereas by the second method a minimum M_r value of 23 500 was found. These findings suggest that, in the undenatured state, transferase X consists of two subunits with M_r 23 500. Transferases X and C migrated together during SDS/polyacrylamide-gel electrophoresis. The subunits of transferase X had R_F values different from those of the known (Hayes *et al.*, 1980) subunits of transferase B, whose M_r values were calculated to be 22 000 (Ya) and 25 000 (Yc) (Figs. 1 and 3b).

Isoelectric focusing of purified enzyme in a pH gradient in the range 5–8 indicated an isoelectric

Fig. 2. Immunoelectrophoresis of purified glutathione *S*-transferases A, C and X and of various mixtures of the three proteins

Antigens were precipitated by either (a) antiserum raised against glutathione *S*-transferase C or (b) antiserum raised against glutathione *S*-transferase X. Antigens: 1, glutathione *S*-transferase X; 2, glutathione *S*-transferases X and C; 3, glutathione *S*-transferase C; 4, glutathione *S*-transferases A and C; 5, glutathione *S*-transferase A; 6, glutathione *S*-transferase X; 7, glutathione *S*-transferases X and A; 8, glutathione *S*-transferase A. Antigens 1–5 were run for 5 h and antigens 6–8 for 2 h at pH 8.6 at 5 V/cm at 4°C.

Fig. 3. Determination of M_r (a) by gel filtration through Sephadex and (b) by SDS/polyacrylamide-gel electrophoresis

Standards were: 1, cytochrome *c* (M_r 12500); 2, chymotrypsinogen (M_r 25000); 3, ovalbumin (M_r 45000); 4, bovine serum albumin (M_r 68000). Arrows show the migration of corresponding purified transferases (X and B). The value for K_{av} on the ordinate of (a) is defined by the equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume of the protein, V_0 is the void volume, determined with ferritin (165 ml), and V_t is the total bed volume, determined from the column dimensions (429 ml).

point at pH 6.9 (Fig. 4). As described by Hayes & Clarkson (1982), analytical isoelectric focusing of the purified preparation of glutathione *S*-transferase A revealed a major and several minor bands, which were reduced to a single band by pretreatment with 2-mercaptoethanol. The same was also observed for the purified preparations of glutathione *S*-transferases C and X, although the minor bands were weaker than those of the glutathione *S*-transferase A preparation. Neither the single band of reduced glutathione *S*-transferase X nor any of the weak additional bands of partially oxidized glutathione *S*-transferase X corresponded to any of the bands of glutathione *S*-transferase A or C.

The amino acid composition of transferase X (Table 2) was similar to that of glutathione *S*-transferase C.

Glutathione *S*-transferase X did not bind cholic

Fig. 4. Isoelectric focusing of glutathione *S*-transferase X. Isoelectric focusing of 5 mg of glutathione *S*-transferase X was performed as described in the Experimental section. Glutathione *S*-transferase activity was monitored with 1-chloro-2,4-dinitrobenzene. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 nmol of product/min under the specific assay conditions.

Table 2. Amino acid composition of glutathione *S*-transferase X and glutathione *S*-transferase C

Analysis involves $\pm 3\%$ error, or for half-cystine $\pm 5\%$. Values represent averages of values obtained after 20 h and 72 h hydrolysis time except for amino acids that are labile under the hydrolysis condition (see footnote *) or relatively resistant to hydrolysis (see footnote †).

Amino acid	Composition (residues/molecule)	
	Transferase X	Transferase C
Lysine	27	27
Histidine	6	5
Arginine	19	18
Aspartate	37	34
Threonine	12*	10*
Serine	14*	14*
Glutamate	45	48
Proline	21	21
Glycine	30	32
Alanine	20	16
Half-cystine	8*	10*
Valine	13†	11†
Methionine	10	10
Isoleucine	13†	13†
Leucine	40	37
Tyrosine	17	18
Phenylalanine	20	18
Tryptophan	6‡	6‡

* Values obtained by extrapolation to zero hydrolysis time.

† Results based on the value after 72 h hydrolysis time.

‡ Determined spectrophotometrically.

Fig. 5. Determination of the cholic acid-binding capacity for (a) glutathione S-transferase B and (b) glutathione S-transferase X

A 17mg portion of partially purified glutathione S-transferase B (5600 units/mg determined with 1-chloro-2,4-dinitrobenzene) or 10 mg of apparently homogeneous glutathione S-transferase X was dialysed against [³H]cholic acid (150 c.p.m./ml) and chromatographed through a Bio-Gel column equilibrated with [³H]cholic acid (150 c.p.m./ml). Radioactivity (○), A₂₈₀ (●) and glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate (Δ) were measured in the column eluate. Glutathione S-transferase activity is expressed as ΔA₃₄₀/min per ml.

acid, whereas transferase B bound cholic acid under identical conditions (Fig. 5).

Substrate specificity

The enzyme assays were not optimized for pH or for substrate and GSH concentrations, but were formed under conditions described in the literature for other glutathione S-transferases. This allows comparison of the results with literature values. Transferase X catalysed the reaction of GSH with all substrates tested except menaphthyl sulphate (Table 3).

Immunological properties

Immunoelectrophoretic analysis of glutathione S-transferases A, C and X and precipitation of various combinations of these antigens by antiserum raised against transferase X and by antiserum raised against transferase C are shown in Fig. 2. When antiserum raised against transferase X was used (Fig. 2b), a continuous precipitation line was observed only with a mixture of transferases X and C, and spur formation was obtained with a mixture of transferases X and A and transferases C and A. When antiserum raised against transferase C was used, a mixture of transferases X and C and transferases A and C gave continuous precipitation lines, whereas precipitation of a mixture of transferases X and A resulted in spur formation. Glutathione transferase X did not react with antiserum raised against either transferase B or E (results not shown).

Table 3. Substrate specificity of glutathione S-transferase X compared with those of glutathione S-transferases C and B

Numbers in parentheses give the corresponding literature values (Jakoby *et al.*, 1976a). Apparently homogeneous preparations of glutathione S-transferases X, C and B were used throughout. Assays were performed as described previously (Habig *et al.*, 1974; Gillham, 1971). Assays were performed in triplicate. Deviations from the mean were less than 10%.

Substrate	Specific activity (units/mg of protein)		
	Transferase X	Transferase C	Transferase B
1-Chloro-2,4-dinitrobenzene	21 500	22 500 (10 000)	16 000 (11 000)
1,2-Dichloro-4-nitrobenzene	2700	1000 (2000)	4 (3)
trans-4-Phenylbut-3-en-2-one	770	470 (400)	<5 (1)
Menaphthyl sulphate	0*	—	6 (4)
1,2-Epoxy-3-(p-nitrophenoxy)-propane	360	280 (0)*	— (0)*
2-(4-Nitrophenyl)-ethyl bromide	180	45	—
p-Nitrobenzyl chloride	3300	(10 200)	(100)

* A value of zero does not necessarily mean that the enzyme is incapable of catalysing the reaction, but rather that the reaction rate was not significantly greater than the spontaneous rate in trials with reasonable amounts of enzyme (> 1 mg of enzyme/assay in our experiments).

Discussion

The present paper describes the isolation and some properties of a highly pure glutathione *S*-transferase (transferase X) that has not previously been observed. Transferases X and M bound to DEAE-cellulose under conditions where transferases AA, A, B, C, D and E did not bind. About 5% of the glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene and 48% of that towards menaphthyl sulphate were retained on DEAE-cellulose. In the next purification step on GSH-Sepharose 4B the activity towards menaphthyl sulphate was separated from that towards 1-chloro-2,4-dinitrobenzene. Thus at least two glutathione *S*-transferase forms were retained on the DEAE-cellulose column, and subsequently separated by affinity chromatography, transferase M and a previously unknown form, which we have called transferase X.

The substrate-specificity of transferase X was different from the known (Jakoby *et al.*, 1976a) substrate-specificities of transferases AA, B, D and E. For example, transferases AA, B, E and also D [as can be concluded from its concentration in the liver (Jakoby *et al.*, 1976b) and the elution pattern of the transferases from a CM-cellulose column monitored with 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane] were nearly inactive in conjugating 1,2-dichloro-4-nitrobenzene (Jakoby *et al.*, 1976a), whereas transferase X conjugated this substrate very rapidly. In addition, no immunological cross-reactivity was observed between transferases B, E and X as determined by immunodiffusion.

Recently Mannervik & Jensson (1982) described a new glutathione *S*-transferase form, C₂. The specific activity of transferase C₂ for 1,2-dichloro-4-nitrobenzene was more than 10-fold lower than that of transferase C (Mannervik & Jensson, 1982); the specific activity of transferase X for this substrate was more than 2-fold higher than that of transferase C (Table 3). In addition, the isoelectric point of glutathione *S*-transferase X was below 7.0, whereas that of transferase C₂ can be estimated from the data presented by Mannervik & Jensson (1982) to be above 8.0.

Transferase X seems closely related to glutathione *S*-transferases A and C. Immunoelectrophoresis with antisera raised against either transferase C or X showed that transferases X, A and C were immunologically similar, but that transferase X is immunologically less related to transferase A than to transferase C. An immunological difference between transferases C and A was not detected in experiments described by Habig *et al.* (1974), with antiserum raised against transferase A. Immunoelectrophoresis showed also that transferases A, C and X had different electrophoretic mobilities. The

main reason for this is a difference in the charge of these proteins. For transferase X we observed an isoelectric point at pH 6.9. Transferases A and C were found in our experiments to have isoelectric points at pH 8.6 and pH 7.9 respectively, whether they were isolated by the method of Habig *et al.* (1974) or as described in the present paper. In addition to their immunological similarities, transferases A, C and X showed similar substrate-specificities, especially towards 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene. The *M_r* of the subunits of transferase X (23 500) was also similar to that of the subunits of glutathione *S*-transferase A or C as described by Hayes *et al.* (1980). They reasoned from their experiments that all glutathione *S*-transferases are dimers composed of subunits with *M_r* 22 000 (Ya) or 23 500 (Yb) or 25 000 (Yc). Cholic acid-binding capacity is restricted to either the Ya or the Yc subunit. Like transferases A and C, transferase X possesses only two Yb subunits and did not bind cholic acid. In contrast, transferase AA (Hayes *et al.*, 1980) and our preparation of transferase B, which contained Ya and Yc subunits, did bind cholic acid.

Thus glutathione *S*-transferase X has properties that differ from those of any other known glutathione *S*-transferase, but it is relatively similar to glutathione *S*-transferases A and C, especially C. It is, however, distinct from glutathione *S*-transferases A and C in its isoelectric point, binding properties to DEAE-cellulose and mobility during immunoelectrophoresis. In addition, treatment of glutathione *S*-transferases A, C and X with 2-mercaptoethanol indicated that glutathione *S*-transferase X does not represent an oxidized or reduced form of either transferase A or C. In addition to the indistinguishable *M_r* values of glutathione *S*-transferases A, C and X observed in the present study, structural investigations by partial sequencing and peptide mapping (Frey *et al.*, 1983) also indicate that glutathione *S*-transferase X does not represent a proteolytic product or precursor of either glutathione *S*-transferase A or C. Glutathione *S*-transferase X appears to be especially important in that we have observed it to be substantially more potent in inactivating a diol epoxide, the vicinal dihydrodiol non-bay-region epoxide *t*-10,11-epoxy-*r*-8,*t*-9-dihydroxy - 8,9,10,11 - tetrahydrobenz[*a*]anthracene, than any of the other major glutathione *S*-transferase forms (A, B and C) (H. R. Glatt & F. Oesch, unpublished work).

Note added in proof (received 14 October 1983)

Our recent results (Frey *et al.*, 1983) indicate that glutathione *S*-transferase X is a homodimer composed of subunit Yb, of the heterodimeric glutathione *S*-transferase C, as is also claimed for glutathione

S-transferase C₂ (Mannervik & Jensson, 1982). However, chromatofocusing of glutathione S-transferase C₂ gave an isoelectric point at pH 8.1 (C. Guthenberg & B. Mannervik, personal communication), confirming non-identity with glutathione S-transferase X. The nature of the structural differences underlying the substantiated functional differences (see also the Discussion section) requires investigation.

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