Different forms of human liver glutathione *S*-transferases arise from dimeric combinations of at least four immunologically and functionally distinct subunits

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Four immunologically distinct subunits were characterized in glutathione (GSH) *S*-transferases of human liver. Five cationic enzymes (pI 8.9, 8.5, 8.3, 8.2 and 8.0) have an apparently similar subunit composition, and are dimers of 26 500- M_r (A) and 24 500- M_r (B) subunits. A neutral enzyme, pI 6.8, is a dimer of B-type subunits. One of the anionic enzymes, pI 5.5, is also a dimer of 26 500- M_r subunits. However, the 26 500- M_r subunits of this anionic enzyme form are immunologically distinct from the A subunits of the cationic enzymes, and have been designated as A'. Immunoabsorption studies with the neutral enzyme, BB, and the antibodies raised against the cationic enzymes (AB) indicate that A and B subunits are immunologically distinct. Hybridization *in vitro* of the A and B subunits of the cationic enzymes (AB) results in the expected binary combinations of AA, AB and BB. Studies with the hybridized enzyme forms indicate that only the A subunits express GSH peroxidase activity. A' subunits have maximum affinity for *p*-nitrobenzyl chloride and *p*-nitrophenyl acetate, and the B subunits have highest activity towards 1-chloro-2,4-dinitrobenzene. The other anionic form, pI 4.5, present in liver is a heterodimer of 22 500- M_r (C) and B subunits. The C subunits of this enzyme are probably the same as the 22 500- M_r subunits present in human lung and placental GSH transferases. The distinct immunological nature of B and C subunits was also demonstrated by immunoaffinity and subunit-hybridization studies. The results of two-dimensional polyacrylamide-gel-electrophoretic analyses indicate that in human liver GSH transferases, three charge isomers of M_r 26 500 (A type), two charge isomers of M_r 24 500 (B type) and two charge isomers of M_r 22 500 (C type) subunits are present.

INTRODUCTION

Glutathione (GSH) *S*-transferases (EC 2.5.1.18) are a family of multifunctional enzymes that catalyse the conjugation of a wide variety of hydrophobic electrophilic xenobiotics to GSH (Booth *et al.*, 1961; Chasseaud, 1979). In addition, some of the forms of GSH *S*-transferase express glutathione peroxidase activity towards lipid hydroperoxides (Prohaska & Ganther, 1977), and some of the members of this enzyme family can bind a number of ligands non-enzymically (Ketterer *et al.*, 1967; Litwack *et al.*, 1971). Through these catalytic and non-catalytic mechanisms, GSH *S*-transferases are believed to provide protection to the tissues from the toxic effects of various xenobiotics, their metabolites and endogenous oxidants such as lipid hydroperoxides. At least eight forms of these enzymes, accounting for about 3% of total soluble protein, are present in human liver (Kamisaka *et al.*, 1975; Awasthi *et al.*, 1980; Warholm *et al.*, 1983). Different forms of human liver GSH transferases have overlapping substrate specificities, and the functional, structural and genetic interrelationships among these forms are not completely understood.

Kamisaka *et al.* (1975) reported the existence of five cationic forms of GSH transferases (α , β , γ , δ and ϵ) in

human liver, and suggested that all of these forms are homodimers and that their charge differences are due to the deamidation of a single gene product. A neutral form, GSH transferase μ , pI 6.6 (Warholm *et al.*, 1983) and two anionic GSH transferases, ω and ψ , pI values 4.5 and 5.5 respectively, have also been reported in human liver (Awasthi *et al.*, 1980). Previous investigations in this laboratory (Dao *et al.*, 1982) indicate that the multiple forms of human liver GSH transferase arise from the binary combinations of three different-size subunits of M_r values 26 500, 24 500 and 22 500. It is not clear if these subunits are three distinct gene products, or result from the post-translational processing of a single gene product. It may also be pointed out that three different subunits can give rise to only six different binary combinations, a number that falls short of explaining the existence of all of the different forms of liver GSH transferase reported so far. Further investigations are therefore needed to elucidate the genetic, structural and functional interrelationships among the multiple forms of human liver GSH transferase.

During the present investigations different forms of GSH transferase of human liver were purified, and their subunit compositions, kinetic properties and immunological properties were determined. By the hybridization

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate; e.l.i.s.a., enzyme-linked immunosorbent assay.

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of the subunits *in vitro* and immunoaffinity techniques, four types of subunits having distinct immunological and kinetic properties were identified in these enzymes.

MATERIALS AND METHODS

Materials

Human liver samples were collected at autopsy from subjects with no diagnosed disorders of the liver. The samples were freed of connective tissue and stored immediately at -20°C until used. The sources of chemicals used were the same as described by us previously (Awasthi *et al.*, 1980; Partridge *et al.*, 1984; Dao *et al.*, 1984).

Enzyme assays

GSH transferase activities with various substrates were determined by the methods described by Habig *et al.* (1974) and Pabst *et al.* (1974). Glutathione peroxidase (EC 1.11.1.9) activity was determined with cumene hydroperoxide as substrate by the method of Awasthi *et al.* (1979). Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Purification of human liver GSH transferases

The results of the present studies were obtained from a single liver sample. However, several such purifications with four different liver samples yielded similar results. Unless otherwise specified, all purification steps were performed at 4°C .

In a typical batch of purification, a 10% (w/v) homogenate of liver was prepared in 10 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer A). The homogenate was centrifuged at 27000 *g* for 45 min, and the supernatant was subjected to affinity chromatography. The GSH was linked to epoxy-activated Sepharose 6B by the method described by Simons & Vander Jagt (1977). The GSH-Sepharose affinity column (1 cm $\times$ 10 cm) was equilibrated with 22 mM-potassium phosphate, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer B) at a flow rate of 10 ml/h, and this rate was maintained during sample application, washing and elution. After application of the enzyme sample, the column was thoroughly washed with buffer B until the eluate had no absorbance at 280 nm. The enzyme was eluted from the column (about 58% yield) with 5 mM-GSH in 50 mM-Tris/HCl, pH 9.6, containing 1.4 mM-2-mercaptoethanol. The eluted enzyme was dialysed against distilled water containing 1.4 mM-2-mercaptoethanol and subjected to isoelectric focusing in an LKB 8100-1 column with Ampholines in the pH range 3.5–10 in a 0–50% (w/v) sucrose density gradient at 1600 V for 18 h. Upon isoelectric focusing two poorly resolved peaks were observed in the range pI 8.0–8.9 along with three distinct peaks at pI 6.8, 5.5 and 4.5 (Fig. 1). The two cationic enzyme peaks were pooled together, dialysed against distilled water containing 1.4 mM-2-mercaptoethanol and subjected again to electrofocusing with Ampholines in the pH range 7–9 (Fig. 2). The enzymes from the remaining three peaks (pI 6.8, 5.5 and 4.5) were also re-electrofocussed separately.

Immunological studies

Antibodies raised against total cationic GSH transferases of human liver and those raised against the anionic

GSH transferases of lung and placenta were the same as those used by us in previous studies (Awasthi *et al.*, 1980; Partridge *et al.*, 1984; Dao *et al.*, 1984). These antibodies were purified by ion-exchange chromatography. The antisera were passed through a column (2 cm $\times$ 10 cm) of DEAE-cellulose (DE-52) pre-equilibrated with buffer A. The unabsorbed material and 1 column volume of washing were pooled, freeze-dried and reconstituted in appropriate volumes for immunological studies.

Preparation of antibodies specific to individual subunits

Antibodies specific to 26500- M_r and 24500- M_r subunits of cationic GSH transferases of human liver were prepared by immunoabsorption methods. The pI 6.8 enzyme form of human liver GSH transferase was found to be a dimer of 24500- M_r subunits. This form was linked to CNBr-activated Sepharose 4B to make the affinity resin. A column (0.5 cm $\times$ 3 cm) of this resin was equilibrated with buffer A, and the antibodies raised in rabbits (Awasthi *et al.*, 1980) against the cationic GSH transferase of human liver (dimers of 24500- M_r and 26500- M_r subunits) were passed through the column. The column was washed with 20 ml of buffer A and eluted with 4 M-KCNS. The unabsorbed antibodies, the washings and the antibodies eluted with KCNS were collected separately and dialysed against buffer A (50 vol., two changes), and tested for immunological cross-reactivity towards various enzymes by using immunodiffusion, immunotitrations and e.l.i.s.a. Immunotitrations were performed by the method described previously by us (Awasthi *et al.*, 1980). Immunodiffusion studies were performed on agar plates (Hyland immuno-plates; Division of Travenol Laboratories, Costa Mesa, CA, U.S.A.) by the method of Ouchterlony (1958).

E.l.i.s.a. was performed by a modification of the method described by Wisdom (1976). Round-bottom 96-well micro-titre plates were coated with the antibodies, which were purified as described in the preceding paragraph by overnight incubation of approx. 5 μg of antibody protein in 150 μl of 0.3 M-borate buffer, pH 8.2, in each well at 37°C . After incubation, the wells were washed with phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.0) and dried. The enzyme samples (250 ng) in 150 μl of phosphate-buffered saline containing 0.5% Tween-20 were then incubated in the wells overnight at 4°C . The wells were gently washed and dried, and 200 μl of reaction mixture for GSH transferase assay (1 mM-GSH and 1 mM-1-chloro-2,4-dinitrobenzene in 100 mM-potassium phosphate buffer, pH 6.5) was added to each well. The change in absorbance of the reaction mixture at 340 nm was recorded at 15 min intervals. In one set of control wells enzyme was omitted, and antibodies were excluded from another set of controls. The non-enzymic increase in absorbance at 340 nm observed in the control wells was subtracted from the increase in absorbance of the reaction mixture in the wells containing the complete system.

Hybridization of subunits *in vitro*

Hybridization of the subunits of different forms of human liver GSH transferase was performed essentially by the method described by other investigators (Kitahara & Sato, 1981; Hayes *et al.*, 1981; Singh *et al.*, 1984). Briefly, the enzyme sample, having 2–4 units of activity, was

incubated with 6 M-guanidinium chloride in 10 ml of 50 mM-potassium phosphate buffer, pH 7.5, containing 20 mM-2-mercaptoethanol at 25 °C for 1 h. The incubation mixture was then diluted 9-fold with 5 mM-potassium phosphate buffer, pH 6.7, containing 0.1 mM-EDTA and 25% (v/v) glycerol, and incubated for an additional 1 h at 25 °C. After the incubation, the reaction mixture was dialysed at 4 °C against the same buffer for 36 h (40 vol., three changes), and the enzyme forms generated during the hybridization *in vitro* were separated by isoelectric focusing as described above under 'Purification of human liver GSH transferases'.

Polyacrylamide-gel electrophoresis

Urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis was performed by the method of Laemmli (1970) with 10%-acrylamide gels in an LKB 2100 vertical apparatus. Two-dimensional electrophoresis studies were performed with the system described by O'Farrell (1975). The gels were stained by the method of Cleveland *et al.* (1977) and destained by the method of Matsudaira & Burgess (1978).

RESULTS

Isolation of human liver GSH transferases

When human liver GSH transferases purified by GSH-Sepharose 6B affinity chromatography were subjected to isoelectric focusing, five peaks of enzyme activity were obtained (Fig. 1). Isoelectric-focusing profiles of GSH transferases present in the 27000 g supernatant of liver used for GSH-Sepharose affinity chromatography were also studied. These profiles were identical with those

Fig. 1. Isoelectric-focusing profile of human liver GSH transferases obtained by affinity chromatography

Experimental details are given in the text. ○, pH gradient; ●, enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate. Inset: urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis of human liver GSH transferases. Lane 1, mixture of cationic enzymes (pI 8.0–8.9); lane 2, neutral enzyme (pI 6.8); lane 3, anionic enzyme (pI 5.5); lane 4, anionic enzyme (pI 4.5).

Fig. 2. Isoelectric-focusing profile of the cationic GSH transferases of human liver with Ampholines of pH range 7–9

Experimental details are given in the text. ○, pH gradient; ●, enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate. Inset: urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis of cationic enzymes separated by electrofocusing. Lane 1, standards in order of decreasing M_r from origin. Lanes 2, 3, 4 and 5 contain GSH transferases having pI values 8.9, 8.5, a mixture of 8.3 and 8.2 and 8.0 respectively.

of the purified enzymes obtained by affinity chromatography. This indicated that during the purification none of the isoenzymes was lost or modified, at least with respect to its charge. Also, during the 8-month period of these investigations, the isoelectric-focusing profiles of the GSH transferases of the liver samples stored at –20 °C remained unaltered. A major portion of human liver GSH transferase activity appeared as two poorly resolved peaks at pH 8.9 and 8.3. When these peaks were pooled together and electrofocused again with Ampholines of a narrow range (pH 7–9), five peaks of GSH transferase activity corresponding to pI values of 8.9, 8.5, 8.3, 8.2 and 8.0 were obtained (Fig. 2). The remaining three peaks of enzyme, having pI values of 6.8, 5.5 and 4.5, were also subjected to re-electrofocusing separately, during which each of them focused as sharp single peak at its original pI.

Subunit structure of human liver GSH transferases

Urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis indicated that all five cationic forms of GSH transferase are heterodimers of 26 500- M_r and 24 500- M_r subunits (Fig. 2 inset). The unequal intensities of 26 500- M_r and 24 500- M_r bands in the gel (Fig. 1) may be due to differential affinity of these subunits for the dye stain. However, the possibility that the cationic enzymes do not represent pure unique heterodimers cannot be ruled out. The neutral form (pI 6.8) is a dimer of 24 500- M_r

subunits (Fig. 1 inset, lane 2), and the less-anionic form (pI 5.5) is a dimer of 26 500- M_r subunits (Fig. 1 inset, lane 3). The more-anionic form (pI 4.5) is a dimer of two non-identical subunits of M_r values 24 500 and 22 500 (Fig. 1 inset, lane 4). On urea/SDS/2-mercaptoethanol/polyacrylamide-gel electrophoresis the mixture of GSH transferases of human liver obtained by affinity chromatography showed the presence of three different-size subunits having M_r values of 26 500, 24 500 and 22 500 (results not shown).

The subunit structures of the five cationic forms correspond to the subunit structures of GSH transferases α , β , γ , δ and ϵ reported previously (Awasthi *et al.*, 1980; Dao *et al.*, 1982). The pI (6.8) of neutral enzyme is close to that reported for GSH transferase μ (Warholm *et al.*, 1983), but the subunit size, M_r 24 500, of this enzyme differs from that reported for GSH transferase μ (M_r 26 500). It may be pointed out that interlaboratory differences in the M_r values of the subunits determined by urea/SDS/polyacrylamide-gel electrophoresis of rat GSH transferases have been reported (Boyer *et al.*, 1983; Hayes, 1984), and it is possible that the neutral enzyme reported here may be similar to GSH transferase μ , because the neutral enzyme also has highest specific activity towards 1-chloro-2,4-dinitrobenzene among all the known GSH transferases of human liver and its specific activities towards other substrates are somewhat similar to those reported for GSH transferase μ .

However, the possibility that these two enzymes are different from each cannot be ruled out from these results. The subunit structure and pI value of the more-anionic form (pI 4.5) may indicate this enzyme to be the same as GSH transferase ω (Awasthi *et al.*, 1980).

Immunological studies

The antibodies raised against the mixture of the cationic forms of human liver GSH transferase, which are heterodimers of 26 500- M_r and 24 500- M_r subunits, cross-reacted with all of the cationic forms. These antibodies also cross-reacted with the neutral form (pI 6.8), and with the more-anionic form, pI 4.5 (Fig. 3*b*). These antibodies, however, did not cross-react with the less-anionic form, pI 4.5 (Fig. 3*b*), indicating that the 26 500- M_r subunits of this enzyme are immunologically distinct from the 26 500- M_r and 24 500- M_r subunits of the cationic enzymes. Our previous studies have shown that anionic GSH transferases of human placenta and lung are homodimers of 22 500- M_r subunits (Partridge *et al.*, 1984; Dao *et al.*, 1984). The antibodies raised against the placental and lung enzymes cross-reacted only with the more-anionic form (pI 4.5) of human liver GSH transferase, indicating that the 22 500- M_r subunits of human placenta and lung enzymes have antigenic determinants common to those present in this anionic form (pI 4.5) of human liver enzyme (Fig. 3*a*). The results of immunological studies obtained by immunotitrations

Fig. 3. Immunotitration of human liver GSH transferases

(a) With antibodies raised against the anionic GSH transferase of lung. (b) With antibodies raised against the mixture of cationic GSH transferases of liver. (c) With antibodies specific to 24 500- M_r subunits of the cationic GSH transferases of liver. (d) With antibodies specific to 26 500- M_r subunits of the cationic GSH transferases of liver. ●—●, Cationic enzyme (pI 8.9); ●—●, neutral enzyme (pI 6.8); ○—○, anionic enzyme (pI 5.5); ○—○, anionic enzyme (pI 4.5). Immunotitration curves determined separately for all the cationic enzymes individually were similar.

Table 1. Immunological characterization of human liver GSH transferases

Immunological cross-reactivities of each of the forms were determined by immunotitrations, double immunodiffusion and e.l.i.s.a. A, 26 500- M_r subunits of cationic enzymes; B, 24 500- M_r subunits of cationic enzymes; C, 22 500- M_r subunits of lung, placenta and liver enzymes.

Antibodies raised against:	Cross-reactivities of the antibodies with human liver GSH transferases			
	Cationic* (pI 8.9)	Neutral (pI 6.8)	Anionic (pI 5.5)	Anionic (pI 4.5)
Anionic GSH transferase of human placenta (CC)	—	—	—	+
Anionic GSH transferases of human lung (CC)	—	—	—	+
Cationic GSH transferases human liver (AB)	+	+	—	+
Antibodies specific to B subunits of cationic human liver GSH transferases	+	+	—	+
Antibodies specific to A subunits of cationic human liver GSH transferases	+	—	—	—

* Immunological properties of other cationic forms were found to be similar.

(Fig. 3) were confirmed by e.l.i.s.a. and double immunodiffusion, and are summarized in Table 1.

Using immunoabsorption techniques we have prepared antibodies that are specific to the 24 500- M_r and 26 500- M_r subunits of the cationic forms of human liver GSH transferase. Since the neutral enzyme (pI 6.8) is a dimer of 24 500- M_r subunits, and it cross-reacts with the antibodies raised against cationic GSH transferases of human liver, it was used to prepare antibodies specific to these two subunits. The antibodies raised against cationic GSH transferases of human liver were passed through a column of CNBr-activated Sepharose 4B to which the neutral enzyme (pI 6.8) was linked. The antibodies that were not absorbed on this column had no cross-reactivity with the neutral enzyme, but still cross-reacted with the cationic enzymes (Fig. 3d). The antibodies absorbed on the column were eluted with 4 M-KCNS. As expected, these antibodies did cross-react with the neutral enzyme (pI 6.8) as well as with the cationic enzymes (Fig. 3c). These antibodies also cross-reacted with the more-anionic enzyme, which also has 24 500- M_r subunits (Fig. 3c).

Hybridization of subunits

When the major cationic form (pI 8.9) of human liver GSH transferase was denatured with 6 M-guanidinium chloride and renatured by removing the denaturing agent, the two subunits of this enzyme associated in the expected binary combinations to yield three active forms of the enzyme, which focused at pI values 8.9, 6.8 and 5.1 (Fig. 4). The subunit structures of these enzymes were determined (Fig. 4 inset). The hybridized enzyme (pI 6.8) was found to be a dimer of 24 500- M_r subunits. The immunological characteristics and substrate specificities of this enzyme were similar to those of the neutral form (pI 6.8) present in human liver (Fig. 4 inset, lane 3, and Tables 2 and 3). The hybridized enzyme (pI 8.9) was found to be a heterodimer of 26 500- M_r and 24 500- M_r subunits (Fig. 4 inset, lane 4), and its properties were the same as those of the cationic form (pI 8.9) of human liver (Tables 2 and 3). The third enzyme (pI 5.1) obtained by hybridization was not similar to any of the enzymes isolated from human liver and was a dimer of 26 500- M_r

subunits (Fig. 4 inset, lane 2). This form cross-reacted with the antibodies raised against cationic GSH transferases of human liver and with the antibodies that were not absorbed on the column of CNBr-activated Sepharose 4B to which either the native neutral enzyme (pI 6.8) or the hybridized enzyme (pI 6.8) was linked.

Fig. 4. Isoelectric-focusing profile of enzymes obtained by hybridizing the subunits of the cationic enzyme (pI 8.9)

Experimental details are given in the text. ○, pH gradient; ●, enzyme activity. Inset: urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis of the hybridized enzymes. Lane 1, standards; lane 2, hybridized enzyme (pI 5.1); lane 3, hybridized enzyme (pI 6.8); lane 4, hybridized enzyme (pI 8.9).

Table 2. Subunit structures and immunological properties of the GSH transferases obtained by hybridization *in vitro*

Antibodies specific to 26 500- M_r (A) and 24 500- M_r (B) subunits of cationic GSH transferases of human liver were prepared as described in the Materials and methods section. Immunological cross-reactivities of each of the enzymes were determined by double immunodiffusion, immunotitrations and e.i.s.a.

Liver enzyme used for hybridization	Enzymes obtained by hybridization (pI)	Cross-reactivity of hybridized enzymes with the antibodies against:			
		Cationic enzymes of liver	Placental enzyme	A subunits	B subunits
Cationic enzyme (pI 8.9, AB)	8.9 (AB)	+	—	+	+
	6.8 (BB)	+	—	—	+
	5.1 (AA)	+	—	+	—
Neutral enzyme (pI 6.8, BB)	6.8 (BB)	+	—	—	+
Anionic enzyme (pI 5.5, A'A')	5.5 (A'A')	—	—	—	—
Anionic enzyme (pI 4.5, BC)	6.8 (BB)	+	—	—	+
	4.5 (BC)	+	+	—	+

Table 3. Substrate-specificities of the GSH transferases obtained by hybridization *in vitro*

One unit of enzyme utilizes 1 μ mol of substrate/min at 25 °C for GSH transferase and at 37 °C for GSH peroxidase. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; *p*-NPA, *p*-nitrophenyl acetate; *p*-NBC, *p*-nitrobenzyl chloride; EAcid, ethacrynic acid; CuOH, cumene hydroperoxide; N.D., not detected.

Enzyme used for hybridization	Hybridized enzymes (pI)	GSH transferase activity (units/mg of protein)					
		CDNB	DCNB	<i>p</i> -NPA	<i>p</i> -NBC	EAcid	CuOH
Cationic enzyme (pI 8.9, AB)	8.9 (AB)	35.2	0.01	0.52	0.52	0.02	1.24*
	6.8 (BB)	49.0	0.025	0.23	0.33	0.08	N.D.
	5.1 (AA)	28.4	0.02	0.89	0.48	0.01	1.44*
Neutral enzyme (pI 6.8, BB)	6.8 (BB)	50.0	0.03	0.22	0.33	0.06	N.D.
Anionic enzyme (pI 5.5, A'A')	5.5 (A'A')	39.0	0.02	1.80	6.2	0.03	N.D.
Anionic enzyme (pI 4.5, BC)	6.8 (BB)	48.6	0.025	0.19	0.30	0.06	N.D.
	4.5 (BC)	24.5	0.12	0.22	1.2	0.04	N.D.

*GSH peroxidase activity at 37 °C.

However, it did not cross-react with the antibodies that were absorbed on these columns and eluted with 4 M-KCNS. This indicates that the antibodies absorbed on the column of neutral enzyme linked to CNBr-activated Sepharose 4B are specific to 24 500- M_r subunits, whereas the antibodies not absorbed on this column are specific to 26 500- M_r subunits of cationic human liver GSH transferases.

Lower pI values of both the homodimers, (26 500- M_r)₂ and (24 500- M_r)₂, observed in this study are surprising. It is possible that associated with the pI 8.9 form is some highly basic component that is removed during the denaturation and renaturation processes. The results of hybridization *in vitro* of the subunits of the pI 8.9 form can also be explained if the subunits, 26 500- M_r and/or 24 500- M_r , of this form represent heterogeneous populations and the more basic subunit(s) do not hybridize *in vitro* to yield homodimer(s). Other possibilities, such as masking of basic charges in the homodimeric combination, should also be considered. Further work with the cationic forms of human liver GSH transferase is needed to resolve these questions, but these results clearly

demonstrate that the 26 500- M_r and 24 500- M_r subunits are immunologically and functionally distinct (Tables 2 and 3).

The hybridization *in vitro* of the subunits of the neutral enzyme (pI 6.8) and the anionic enzyme (pI 5.5), which are holodimers of 24 500- M_r and 26 500- M_r respectively, was also carried out. Hybridization of the subunits of the pI 6.8 and the pI 5.5 forms followed by isoelectric focusing both gave single sharp peaks of enzyme activity at their parent pI values (results not shown). The more-anionic form (pI 4.5) is a heterodimer of 24 500- M_r and 22 500- M_r subunits, and upon hybridization was expected to yield three enzymically active dimers having subunit compositions of (24 500- M_r)₂, (24 500- M_r -22 500- M_r) and (22 500- M_r)₂. However, only two peaks of hybridized enzymes were obtained upon isoelectric focusing of the reaction mixture after denaturation and renaturation of the subunits of the anionic enzyme, pI 4.5 (Fig. 5). The hybridized enzyme form, pI 6.8, had similar immunological properties and substrate specificities to those of the neutral enzyme, pI 6.8, and as expected was found to be a homodimer of

Fig. 5. Isoelectric-focusing profile of reconstituted enzymes obtained by hybridizing subunits of anionic enzyme (pI 4.5)

Experimental details are given in the text. ○, pH gradient; ●, enzyme activity. Inset: urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis of hybridized enzymes. Lane 1, standards; lane 2, hybridized enzyme (pI 4.5); lane 3, hybridized enzyme (pI 6.8).

24 500- M_r subunits (Fig. 5 inset, lane 3). This finding strongly suggests that 24 500- M_r subunits present in the anionic enzyme, pI 4.5, the neutral enzyme, pI 6.8, and the cationic enzymes, pI 8.0–8.9, are similar. The other hybridized enzyme, having pI 4.5 (Fig. 5), was found to be a heterodimer of 24 500- M_r and 22 500- M_r subunits (Fig. 5 inset, lane 2) and was similar to the parent enzyme in all of the properties studied (Tables 2 and 3). The third expected enzyme peak corresponding to the binary combination of (22 500- M_r)₂ was not observed during isoelectric focusing. However, it may be pointed out that the anionic enzyme of human placenta, which has been demonstrated to be a dimer of 22 500- M_r subunits (Dao *et al.*, 1984), also has an isoelectric point of about 4.5, and it is possible that the peak of the hybridized enzyme at pH 4.5 may actually represent a mixture of enzyme forms having the subunit compositions of (22 500- M_r)₂ and (24 500- M_r -22 500- M_r).

Immunological characterization of 22 500- M_r and 24 500- M_r subunits of the anionic enzyme, pI 4.5, was carried out by means of competitive-inhibition studies in the immunotitrations. The enzyme, pI 4.5, obtained by the hybridization of the subunits of anionic enzyme (pI 4.5) was passed through a column of CNBr-activated Sepharose 4B to which antibodies raised against the placental enzyme were linked. The column was successively eluted with 3 M-urea and 4 M-KCNS. Urea/SDS/2-mercaptoethanol/polyacrylamide-gel electrophoresis of the fraction eluted with urea showed the presence of only 24 500- M_r subunits. On the other hand the KCNS eluate showed the presence of only 22 500- M_r subunits. These fractions were used to study the competitive inhibition in immunotitration studies. The anionic GSH transferase from human placenta was partially purified by using

Fig. 6. (a) Immunotitration of placental GSH transferase with antibodies raised against placental GSH transferase, and (b) immunotitration of neutral human liver GSH transferase (pI 6.8) with antibodies specific to 24 500- M_r subunits of cationic enzyme (pI 8.9)

Details are given in text. Inset to (a): the reaction mixture was preincubated with the urea eluate (24 500- M_r subunits) (○-○) and with the KCNS eluate (22 500- M_r subunits) (○-○), which were obtained by passing the anionic enzyme (pI 4.5) over an immunoaffinity column to which the antibodies raised against the placental enzyme were linked. Inset to (b): the reaction mixture was preincubated with the KCNS eluate (22 500- M_r subunits) (○-○) and with the urea eluate (24 500- M_r subunits) (○-○).

Table 4. Substrate-specificities of human liver GSH transferases

One unit of enzyme utilizes 1 μ mol of substrate/min at 25 °C for GSH transferase and at 37 °C for GSH peroxidase. Abbreviation: N.D., not detectable.

Substrate	GSH transferase activity (units/mg of protein)			
	Cationic (pI 8.9)	Neutral (pI 6.8)	Anionic (pI 5.5)	Anionic (pI 4.5)
1-Chloro-2,4-dinitrobenzene	16.75	54.0	40.2	27.7
1,2-Dichloro-4-nitrobenzene	0.01	0.03	0.02	0.10
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	1.2	0.12	N.D.	0.18
Ethacrynic acid	0.02	0.08	0.04	0.02
Bromosulphophthalein	0.04	0.01	N.D.	N.D.
<i>p</i> -Nitrophenyl acetate	0.70	0.24	1.40	0.23
<i>p</i> -Nitrobenzyl chloride	0.56	0.48	5.0	2.4
<i>trans</i> -4-Phenylbut-3-en-2-one	0.18	0.40	N.D.	0.10
Cumene hydroperoxide*	1.3	N.D.	N.D.	N.D.

*GSH peroxidase activity at 37 °C.

DEAE-cellulose chromatography, and the immunotitration curve was obtained by titrating with antibodies raised against homogeneous placental enzyme. From the immunotitration curve an amount of antibody was selected that caused about 55% inhibition (Fig. 6*a*). During the immunotitration of placental enzyme with its own antibodies, the immunoprecipitation was inhibited by addition of the KCNS eluate (22 500- M_r subunits), but not by addition of the urea eluate (24 500- M_r subunits), as seen in Fig. 6(*a*) inset. This indicates that the 22 500- M_r subunits of the anionic form (pI 4.5) have immunogenic determinants similar to those present in the 22 500- M_r subunits of the placental enzyme, and that the 24 500- M_r and 22 500- M_r subunits of liver enzyme are immunologically distinct from each other. To substantiate this contention further in a separate experiment the neutral enzyme was titrated with the antibodies specific to 24 500- M_r subunits prepared by immunoabsorption techniques. A volume of antibody was selected that brought about 55% inhibition of the enzyme (Fig. 6*b*). Addition of the urea eluate, which contains 24 500- M_r subunits of the anionic form, pI 4.5, caused inhibition of the immunoprecipitation of the neutral enzyme, pI 6.8, with antibodies specific to 24 500- M_r subunits (Fig. 6*b* inset). However, addition of the KCNS eluate, which contains 22 500- M_r subunits, did not inhibit this immunoprecipitation. These results further confirm the distinct immunogenic natures of 22 500- M_r and 24 500- M_r subunits of liver enzymes.

Substrate-specificities

The substrate-specificities of the enzymes isolated from human liver and the enzymes formed by hybridization *in vitro* of the subunits of liver enzymes are presented in Tables 4 and 3 respectively. These results indicate that the four types of immunologically distinct subunits characterized in the present study have different affinities towards different substrates. Glutathione peroxidase II activity towards cumene hydroperoxide is associated only with the 26 500- M_r subunits of the cationic GSH transferases (pI 8.0–8.9), since only these forms and the hybridized enzyme form (pI 5.1) express this activity. The other type of 26 500- M_r subunits present in the anionic

enzyme, pI 5.5, do not express this activity. The subunits of this enzyme, however, appear to have high specific activities towards *p*-nitrophenyl acetate and *p*-nitrobenzyl chloride. High specific activities towards 1-chloro-2,4-dinitrobenzene, ethacrynic acid and *trans*-4-phenylbut-3-en-2-one are displayed by the 24 500- M_r subunits. The 22 500- M_r subunits appears to have relatively higher specific activity towards 1,2-dichloro-4-nitrobenzene.

Fig. 7. Two-dimensional polyacrylamide-gel electrophoresis of human liver GSH transferases

(*a*) Affinity-purified; (*b*) mixture of cationic enzymes; (*c*) less-anionic enzyme, pI 5.5; (*d*) more-anionic enzyme, pI 4.5.

Two-dimensional-electrophoresis studies

When the mixture of all of the GSH transferases of human liver obtained by affinity chromatography was subjected to two-dimensional electrophoretic analysis, at least seven distinct polypeptide spots were observed on the gel (Fig. 7a). Three of these spots appeared corresponding to M_r values of 26 500, whereas two spots each were seen corresponding to M_r values of 24 500 and 22 500. When a mixture of all the five cationic enzymes, pI 8.9, 8.5, 8.3, 8.2 and 8.0, was subjected to two-dimensional gel electrophoresis, four spots, two each corresponding to M_r values of 26 500 and 24 500, were observed (Fig. 7b). The less-anionic enzyme (pI 5.5) showed the presence of a single spot, corresponding to an M_r of 26 500 (Fig. 7c), whereas the more-anionic enzyme had two spots corresponding to 22 500- M_r subunits and two spots corresponding to 24 500- M_r subunits (Fig. 7d).

DISCUSSION

The present investigations provide convincing evidence that there are at least four distinct subunits present in GSH transferases of human liver. These subunits are immunologically dissimilar from each other and differ in their kinetic characteristics. Two of these subunits apparently have the same M_r value of 26 500, and we have designated these subunits as A and A'. The other two subunits, designated as B and C, have M_r values of 24 500 and 22 500 respectively.

The five cationic enzymes of human liver have an apparently similar subunit composition and are heterodimers of 26 500- M_r (A) and 24 500- M_r (B) subunits. The antibodies raised against a mixture of these forms cross-react with the neutral form, pI 6.8, and the anionic form, pI 4.5, both of which have 24 500- M_r subunits, indicating that the 24 500- M_r subunits of these two forms may be similar to those of the cationic enzymes. The antibodies raised against the cationic enzymes, however, do not recognize the immunogenic determinants on the 26 500- M_r subunits of the less-anionic form, pI 5.5, indicating that these subunits are immunologically distinct from both A and B subunits of the cationic enzymes. The subunit structure of this enzyme form (pI 5.5) is designated as A'A', and this form has not been characterized previously from human liver.

Immunoabsorption studies with the neutral enzyme (BB) and the antibodies raised against the cationic enzymes (AB) provide evidence for the distinct immunological natures of A and B subunits. This evidence is further substantiated by the immunoabsorption studies with the AA and BB homodimers obtained by hybridization *in vitro* of the subunits of the cationic enzyme (AB). Distinct immunological nature of the 22 500- M_r subunits is indicated by the fact that the antibodies raised against the placental enzyme (CC) do not recognize the antigenic determinants on the cationic (AB), the neutral (BB) or the less-anionic enzyme (A'A'). Also, the antibodies specific to either A or B subunits do not recognize antigenic determinants on the placental enzyme, CC (results not shown). The results of competitive-inhibition studies in immunotitrations with the inactive preparations of B and C subunits further substantiate the distinct immunological nature of B and C subunits. Taken together, these results strongly suggest that A (26 500- M_r), A' (26 500- M_r), B (24 500- M_r) and C

(22 500- M_r) subunits are immunologically and structurally distinct and are possibly the products of different genes.

Four subunits in their binary combinations can provide the structural basis for up to ten different forms of GSH transferase in human liver. However, because of the apparently similar subunit compositions of the five cationic GSH transferases (α , β , γ , δ and ϵ), these studies do not provide complete understanding of the underlying structural basis for the multiple forms of GSH transferase present in human liver. In particular, the interrelationship among these five forms of apparently similar subunit structure is not explained by these studies. It is possible that these forms arise by deamidation *in vivo* of a single form, as suggested by Kamisaka *et al.* (1975). On the other hand, the presence of as yet uncharacterized distinct subunits having M_r values similar to those of A and B subunits could also explain the differences in the characteristics of α , β , γ , δ and ϵ . Two-dimensional electrophoretic analysis indicates the presence of three types of A subunits, two types of B subunits and two types of C subunits in human liver GSH transferases, suggesting a considerable amount of heterogeneity in the A-, B- and C-type subunits. Such a heterogeneity could explain the differences in all known GSH transferases of human liver on a structural basis. If the seven different polypeptides indicated by two-dimensional gel-electrophoretic analysis are indeed seven different subunits, the presence of a multitude of 28 possible GSH transferase forms can be predicted to exist in human liver. Through their diverse functionalities, these forms can provide human tissues with efficient mechanisms of protection from xenobiotics, their metabolites and oxidants generated *in vivo*.

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