Interrelationship between anionic and cationic forms of glutathione S-transferases of human liver

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(Received 13 November 1979/Accepted 9 June 1980)

Human liver glutathione S-transferases (GSH S-transferases) were fractionated into cationic and anionic proteins. During fractionation with $(\text{NH}_4)_2\text{SO}_4$ the anionic GSH S-transferases are concentrated in the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction, whereas the cationic GSH S-transferases separate in the 80%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction. From the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction two new anionic GSH S-transferases, ω and ψ , were purified to homogeneity by using ion-exchange chromatography on DEAE-cellulose, Sephadex G-200 gel filtration, affinity chromatography on GSH bound to epoxy-activated Sepharose and isoelectric focusing. By a similar procedure, cationic GSH S-transferases were purified from the 80%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction. Isoelectric points of GSH S-transferases ω and ψ are 4.6 and 5.4 respectively. GSH S-transferase ω is the major anionic GSH S-transferase of human liver, whereas GSH S-transferase ψ is present only in traces. The subunit mol.wt. of GSH S-transferase ω is about 22 500, whereas that of cationic GSH S-transferases is about 24 500. Kinetic and structural properties as well as the amino acid composition of GSH S-transferase ω are described. The antibodies raised against cationic GSH S-transferases cross-react with GSH S-transferase ω . There are significant differences between the catalytic properties of GSH S-transferase ω and the cationic GSH S-transferases. GSH peroxidase II activity is displayed by all five cationic GSH S-transferases, whereas both anionic GSH S-transferases do not display this activity.

Glutathione S-transferases (GSH S-transferases) represent a family of enzymes (EC 2.5.1.18) that catalyse the conjugation to glutathione of a large group of hydrophobic compounds bearing an electrophilic centre (Booth *et al.*, 1961; Habig *et al.*, 1974a). Some of these proteins are also designated ligandin (Litwak *et al.*, 1971; Arias, 1972; Habig *et al.*, 1974b) and bind non-catalytically certain hydrophobic compounds such as bilirubin, and are believed to assist in the solubilization and storage of such hydrophobic molecules in liver (Wolkoff *et al.*, 1978). They also bind covalently to certain strong alkylating agents such as azo-dye carcinogens and are believed to offer protection to proteins and other macromolecules from these agents (Ketterer *et al.*, 1967; Jakoby, 1978).

Seven GSH S-transferases have been isolated

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

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from rat liver (Gillham, 1971, 1973; Pabst *et al.*, 1973, 1974; Habig *et al.*, 1974a,b; Jakoby, 1978), and at least five GSH S-transferases designated as GSH S-transferases α , β , γ , δ and ϵ have been reported from human liver (Kamisaka *et al.*, 1975). All GSH S-transferases reported so far from human liver (pI 7.8–8.8) are cationic in nature and are immunologically similar (Kamisaka *et al.*, 1975; Jakoby, 1978). However, there appear to be three immunologically distinct species of GSH S-transferases in rat liver (Jakoby, 1978). Yet another transferase, designated GSH S-transferase ρ , has been reported from human erythrocytes (Marcus *et al.*, 1978). In contrast with liver enzymes, GSH S-transferase ρ has an acidic pI and is immunologically distinct.

GSH S-transferase of rat liver has been shown to express selenium-independent glutathione peroxidase activity (GSH peroxidase II) directed only towards organic hydroperoxides such as cumene hydroperoxide (Lawrence & Burk, 1976; Prohaska & Ganther, 1977). This appears to implicate GSH

S-transferases in an additional physiological role of protection against oxidative stress.

During the present investigation two additional GSH S-transferases have been found in human liver. Unlike other GSH S-transferases of human liver, these transferases are anionic proteins. Structural, kinetic and immunological properties of one of these components, which constitutes the bulk of anionic GSH S-transferases, have been studied.

Materials and methods

Materials

Human liver samples were collected at autopsy from the subjects of diseases unrelated to liver disorders. The samples were freed of connective tissues and processed immediately. Sephadex G-200 and G-150, epoxy-activated Sepharose 6B, GSH, NADPH, glutathione reductase (yeast, type III), 1-chloro-2,4-dinitrobenzene and *N*-ethylmaleimide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman Co., Maidstone, Kent, U.K. Ampholines were obtained from LKB Produkter, Bromma, Sweden. Cumene hydroperoxide and *t*-butyl hydroperoxide were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. *p*-Nitrobenzyl chloride and 1,2-dichloro-4-nitrobenzene were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 4-nitropyridine *N*-oxide were kindly provided by Dr. W. B. Jakoby, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A. Goat antisera against rabbit γ -globulins were kindly provided by Dr. Philip Rayford, Surgical Biochemistry Laboratory, University of Texas Medical Branch, Galveston, TX, U.S.A.

Enzyme assay

The enzyme assays for GSH peroxidase with *t*-butyl hydroperoxide and cumene hydroperoxide were performed by the procedure of Awasthi *et al.* (1975), except that 0.1 M-Tris/HCl buffer, pH 7.0, was used instead of phosphate buffer. The rate of reaction was measured by following the decrease in A_{340} corresponding to the oxidation of NADPH in a Gilford model 2400-2 spectrophotometer at 37°C. The iodometric titration by the method of Kokatnur & Jelling (1941) was used to determine the exact concentrations of the stock solutions of *t*-butyl hydroperoxide and cumene hydroperoxide. To study the inhibition of GSH peroxidase activity by cyanide, the enzyme was incubated at 4°C for 30 min with 33.3 mM-KCN. After incubation, a 10 μ l portion of the incubation mixture was assayed for GSH peroxidase activity.

GSH S-transferase was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with GSH as described by Habig *et al.* (1974a). The assay mixture contained (in 1 ml) 1 mM-1-chloro-2,4-dinitrobenzene, 1 mM-GSH and 0.1 M-potassium phosphate buffer, pH 6.5; the rate of increase in A_{340} was monitored at 25°C in a Gilford model 2400-2 spectrophotometer for 5–10 min after the addition of appropriate amounts of enzyme. During this period the reaction rate was linear with time. The enzyme assays with other substrates such as *p*-nitrobenzyl chloride, 4-nitropyridine *N*-oxide, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 1,2-dichloro-4-nitrobenzene were performed by methods described previously (Booth *et al.*, 1961; Pabst *et al.*, 1973; Fjellstedt *et al.*, 1973). The unit of enzyme activity was defined as 1 μ mol of substrate utilized/min at 37°C for GSH peroxidase and at 25°C for GSH S-transferase.

Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis (1964), and the gels were stained for protein by the procedure of Reisner *et al.* (1975) and destained by a rapid-destain procedure (Saneto *et al.*, 1980). Urea/SDS/ β -mercaptoethanol/polyacrylamide-disc-gel electrophoresis was performed as described by Laemmli (1970). Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard. Haemoglobin concentrations in the homogenate and 10000g supernatant were measured by adding homogenate to Drabkin's solution [containing 100 mg of NaCN and 300 mg of $K_3Fe(CN)_6$ per litre] to convert the pigment into cyanmethaemoglobin and then determining the A_{540} in a Coleman Junior model 6C spectrophotometer as described by Beutler (1975).

Amino acid analysis

Determination of the amino acid composition of purified GSH S-transferase was performed with a Beckman model 121M automatic amino acid analyser. A 50 μ g sample of the enzyme was hydrolysed *in vacuo* in 500 μ l of 5.7 M-HCl for 24, 48 and 96 h. The samples were then dried *in vacuo* over NaOH, dissolved in 150 μ l of 300 mM-lithium citrate buffer, and 50 μ l samples applied to the column for analysis.

Antibodies against cationic GSH S-transferases

About 80 μ g of purified cationic GSH S-transferases mixed in 1 ml of Freund's adjuvant (complete) was injected subcutaneously into a rabbit, followed by a booster injection after 2 weeks. The rabbit was bled 4 weeks after the first injection, and the antiserum obtained was heat-inactivated at 55°C for 4 h. Attempts to raise antibodies against GSH S-transferase ω in a similar manner were unsuccessful. Immunodiffusion studies were performed on agar plates (Hyland immuno-plate; Div

Travenol Laboratory, Costa Mesa, CA, U.S.A., Pattern C). Immunotitration studies were done by incubating about 50 munits of purified GSH *S*-transferases (corresponding to about 3.75 μ g of protein) in a 0.25 ml system in 10 mM-potassium phosphate buffer, pH 7.0, with the antiserum overnight at 4°C. Then 10 μ l of goat anti-(rabbit immunoglobulin G) serum was added and the mixture was further incubated at 4°C for 30 min. Subsequently the reaction mixture was centrifuged at 20000 *g* for 1 h, and GSH *S*-transferase activity was measured in the supernatant, with 1-chloro-2,4-dinitrobenzene as substrate.

Purification of GSH *S*-transferases

The results described below were obtained with a single human liver. Similar results were obtained during three separate purifications by using liver samples from unrelated subjects. Unless otherwise indicated, all steps were performed at 4°C. Human liver was cleaned and washed thoroughly with cold deionized distilled water. After being cut into small pieces, the liver (100 g) was homogenized in 5 mM-potassium phosphate buffer, pH 7.0 (1 litre), containing 1.4 mM- β -mercaptoethanol. The homogenate was centrifuged at 10000 *g* for 45 min and the clear supernatant was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the supernatant to give 65% saturation. The mixture was stirred overnight and centrifuged at 10000 *g* for 1 h. By further addition of solid $(\text{NH}_4)_2\text{SO}_4$, the supernatant was made 80%-saturated. The mixture was stirred overnight and pellet was collected by centrifugation at 10000 *g* for 1 h. The supernatant, which had negligible GSH *S*-transferase and GSH peroxidase activities, was discarded. Pilot experi-

ments of ion-exchange chromatography on DEAE-cellulose (DE-52) and CM-cellulose (CM-52) columns indicated that the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction was rich in anionic GSH *S*-transferases, whereas the 80%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction consisted almost exclusively of the cationic GSH *S*-transferases. Thus GSH *S*-transferases from both these fractions were purified separately (Table 1).

(a) *Purification of anionic GSH S-transferases.* The pellet obtained by 65% saturation with $(\text{NH}_4)_2\text{SO}_4$ and centrifugation was resuspended in 5 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM- β -mercaptoethanol, and was dialysed against the same buffer for 72 h (4×50 vol.). The dialysed enzyme was centrifuged at 10000 *g* for 45 min and the supernatant was passed through a CM-cellulose (CM-52) column (2.5 cm $\times$ 40 cm) pre-equilibrated with 5 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM- β -mercaptoethanol, at 40 ml/h. The anionic GSH *S*-transferase did not bind to the column. The eluate and wash were pooled and subjected to DEAE-cellulose (DE-52) chromatography.

DEAE-cellulose (DE-52) chromatography. The unadsorbed enzyme fraction after CM-cellulose (CM-52) chromatography was dialysed against 5 mM-potassium phosphate buffer, pH 7.2, containing 1.4 mM- β -mercaptoethanol and was passed through a DEAE-cellulose (DE-52) column (2.5 cm $\times$ 40 cm) that had been pre-equilibrated with the same buffer, at 40 ml/h. Almost all of the enzyme was adsorbed on the column. After the column had been washed with the equilibrating buffer, the enzymes were eluted with a 800 ml linear gradient of 0–200 mM-NaCl in the application buffer. The

Table 1. *Purification of GSH S-transferases from human liver*
Experimental details are given in the text.

	Activity		Protein (mg/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
	(units/ml)	(total units)				
Homogenate	1.15	1260.0	10.26	0.11	—	100
65%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction	0.96	182.0	16.0	0.06	0.5	14
80%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction	8.21	853.0	10.08	0.81	7.4	68
Anionic GSH <i>S</i> -transferase						
65%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction	0.96	182.0	16.00	0.06	0.5	100
CM-cellulose CM-52	0.52	140.0	8.95	0.06	0.5	77
DEAE-cellulose DE-52	0.32	85.0	1.05	0.30	2.7	46
Sephadex G-200	0.45	63.0	1.03	0.44	4.0	34
Affinity chromatography	0.75	25.5	0.07	10.71	97.4	14
Electrofocusing	0.78	14.0	0.03	26.00	236.4	8
Cationic GSH <i>S</i> -transferase						
80%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction	8.21	853.0	10.08	0.81	7.4	100
CM-cellulose CM-52	1.75	367.0	0.50	3.50	31.8	43
Affinity chromatography	1.63	94.5	0.30	5.43	49.4	11
Electrofocusing	1.06	46.7	0.075	14.13	128.5	5

enzyme was eluted as a single broad peak between 30 mM- and 80 mM-NaCl. The pooled fractions from the DEAE-cellulose column were concentrated to 60 ml in an Amicon ultrafiltration cell with a PM-10 membrane and subjected to Sephadex G-200 gel filtration.

Fig. 1. *Sephadex G-200 gel filtration of anionic GSH S-transferases and GSH peroxidase I*

Experimental details are given in the text. Fractions of volume 5 ml were collected. ▲, GSH S-transferase activity; ■, GSH peroxidase activity with t-butyl hydroperoxide; ○, GSH peroxidase activity with cumene hydroperoxide.

Fig. 2. *Isoelectric focusing of anionic GSH S-transferases*

Details are described in the text. ○, pH gradient; ●, GSH S-transferase activity.

Sephadex G-200 gel filtration. The Sephadex G-200 column (5 cm × 100 cm) was equilibrated with 5 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-β-mercaptoethanol and 100 mM-(NH₄)₂SO₄. The pooled DEAE-cellulose fraction after concentration was passed through the column by using upward flow at 60 ml/h. During gel filtration, GSH peroxidase and GSH S-transferase separated from each other (Fig. 1) as two distinct peaks. At this stage, the GSH peroxidase and GSH S-transferase were pooled separately. The pooled fraction containing GSH S-transferase activity was further purified by affinity chromatography, whereas GSH peroxidase was further purified by the method described by Awasthi *et al.* (1979).

Affinity chromatography. The GSH affinity resin was prepared by linking GSH to epoxy-activated Sepharose 6B by the method described by Simons &

Fig. 3. *Polyacrylamide-disc-gel electrophoresis of GSH S-transferases*

Details are described in the text. (a) GSH S-transferase ω in the system of Davis (1964). (b) Urea/SDS/polyacrylamide-disc-gel electrophoresis of a mixture of GSH S-transferase ω and cationic GSH S-transferase. Arrows: A, subunit of cationic GSH S-transferase; B, subunit of GSH S-transferase ω.

Vander Jagt (1977). The GSH affinity column (1 cm × 10 cm) was equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, at 10 ml/h. After dialysis against the same buffer, the GSH *S*-transferase fraction was applied to the affinity column and the column was washed thoroughly with the application buffer. The enzyme was eluted with 50 mM-Tris/HCl buffer, pH 9.6, containing 5 mM-GSH. The eluate fractions containing activity were pooled, dialysed against deionized distilled water containing 0.1 mM- β -mercaptoethanol and subjected to isoelectric focusing.

Isoelectric focusing. Isoelectric focusing of anionic GSH *S*-transferase from the affinity-chromatography step was performed on a LKB model 8100-1 column by using Ampholines in the pH range 3.5–10 in order to detect any contaminating cationic GSH *S*-transferases. The elution pattern (Fig. 2) shows two peaks of enzyme, with pI values 4.6 and 5.4 respectively. The major peak (pI 4.6) accounted for almost all the GSH *S*-transferase activity, whereas the minor peak (pI 5.4) represented only traces of enzyme activity. Both peaks were pooled separately. All the kinetic and structural studies were performed with the major anionic GSH *S*-transferase, designated as GSH *S*-transferase ω .

The results of purification are presented in Table 1. GSH *S*-transferase ω appeared as a sharp single band during polyacrylamide-disc-gel electrophoresis (Fig. 3a). The purity of the trace component GSH *S*-transferase ψ could not be ascertained owing to paucity of material.

(b) *Purification of cationic GSH S-transferase.* The 80%-saturated-(NH₄)₂SO₄ fraction was resuspended in 5 mM-potassium phosphate buffer, pH 7.0,

containing 1.4 mM- β -mercaptoethanol and dialysed against the same buffer for 72 h (8 × 100 vol.). The dialysed enzyme was then centrifuged at 10000 g for 45 min and the pellet was discarded.

CM-cellulose (CM-52) column chromatography. The supernatant was passed through a CM-cellulose (CM-52) column (2.5 cm × 40 cm) pre-equilibrated with 5 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM- β -mercaptoethanol at 40 ml/h. After the column had been washed with the application buffer, the enzymes were eluted with a 800 ml linear gradient of 0–200 mM-NaCl in the same buffer. GSH *S*-transferases were eluted between 10 mM- and 50 mM-NaCl as several peaks, and GSH peroxidase activity was also associated with these peaks. All the peaks containing enzyme activity were pooled together and subjected to GSH affinity-column chromatography as described above for the anionic GSH *S*-transferases. The enzyme obtained after affinity chromatography had activities of both GSH *S*-transferase and GSH peroxidase (Table 2).

Isoelectric focusing. The enzyme obtained from the GSH affinity column was dialysed against deionized distilled water and subjected to column electrofocusing (LKB model 8100-1 column) in a 0–50% (w/v) sucrose density gradient by using Ampholines in the pH range 3.5–10.0. Ampholines of wide pH range were used specially to ascertain if GSH *S*-transferase ω (pI 4.6) was present in these fractions. Five poorly resolved peaks of GSH *S*-transferase were obtained, having pI values in the range 8.5–9.5. GSH peroxidase activity (GSH peroxidase II) was associated with all the fractions having GSH *S*-transferase activity. However, the peaks of two activities were not exactly super-

Table 2. *Ratio of GSH S-transferase and GSH peroxidase activity during the purification of GSH S-transferases*
GSH peroxidase activity was determined with cumene hydroperoxide as substrate. For full details see the text.

	GSH peroxidase (units/mg of protein)	GSH <i>S</i> -transferase (units/mg of protein)	GSH peroxidase/GSH <i>S</i> -transferase activity ratio
Homogenate	0.12	0.11	1.09
65%-satd.-(NH ₄) ₂ SO ₄ fraction	0.18	0.06	3.00
80%-satd.-(NH ₄) ₂ SO ₄ fraction	0.51	0.81	0.62
Anionic GSH <i>S</i> -transferase			
65%-satd.-(NH ₄) ₂ SO ₄ fraction	0.18	0.06	3.00
CM-cellulose (CM-52) unadsorbed	0.32	0.06	5.30
DEAE-cellulose (DE-52)	4.65	0.30	15.50
Sephadex G-200	0.02	0.44	0.05
Affinity chromatography	0.00	10.71	0.00
Preparative isoelectrofocusing	0.00	26.00	0.00
Cationic GSH <i>S</i> -transferase			
80%-satd.-(NH ₄) ₂ SO ₄ fraction	0.51	0.81	0.62
CM-cellulose	1.80	3.50	0.51
Affinity chromatography	2.73	5.43	0.50
Preparative isoelectrofocusing	5.07	14.13	0.36

Fig. 4. Isoelectric focusing of cationic GSH *S*-transferases

Details are described in the text. ●, GSH *S*-transferase activity; ▲, GSH peroxidase II activity; ○, pH gradient.

impossible (Fig. 4). Rather than being pooled separately, all GSH *S*-transferase peaks were pooled together. The pooled GSH *S*-transferases appeared as a single peak of mol.wt. about 46 000 on a Sephadex G-150 gel-filtration column. Also, urea/SDS/ β -mercaptoethanol/polyacrylamide-disc-gel electrophoresis showed the presence of only one protein band, indicating that probably the preparation represents only the GSH *S*-transferase having a subunit size of about 24 500 daltons.

Results

There are at least two anionic species of GSH *S*-transferases present in human liver. They constitute about 10% of the total GSH *S*-transferase of human liver. The major GSH *S*-transferase, designated here as GSH *S*-transferase ω , accounts for almost all of the anionic GSH *S*-transferases, whereas the other enzyme (GSH *S*-transferase ψ) is present only in trace amounts.

In the purification procedure the anionic GSH *S*-transferases separate from the cationic GSH *S*-transferases during the fractionation with $(\text{NH}_4)_2\text{SO}_4$. The anionic enzymes are almost completely precipitated in the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction, whereas the cationic enzymes are almost exclusively present in the 80%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction. This early fractionation of the two activities is very helpful for their further purification separately. Also during this fractionation, selenium-dependent GSH peroxidase activity is precipitated along with anionic GSH *S*-transferases, and separates from the cationic GSH *S*-transferases. The anionic GSH *S*-transferases (ω and ψ) in the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction are contami-

nated with only small amounts of cationic GSH *S*-transferase, which are effectively separated by CM-cellulose column chromatography. The activity of GSH *S*-transferase not bound to CM-cellulose at this stage represents almost exclusively the anionic GSH *S*-transferases. DEAE-cellulose column chromatography further separates the remaining contamination of cationic GSH *S*-transferases in the anionic GSH *S*-transferases fraction. The resolution of GSH *S*-transferases ω and ψ on DEAE-cellulose chromatography at this stage is not achieved, and instead a broad peak of GSH *S*-transferase activity is obtained. GSH peroxidase activity associated with the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction co-purified with anionic GSH *S*-transferases until the DEAE-cellulose chromatography step. GSH peroxidase activity associated with these fractions, however, is cyanide-sensitive, is selenium-dependent and is expressed towards H_2O_2 as well as cumene hydroperoxide or *t*-butyl hydroperoxide as substrates, and therefore, probably expressed by the selenoprotein GSH peroxidase I (Lawrence & Burk, 1976; Awasthi *et al.*, 1979). During the gel filtration through Sephadex G-200 the two activities segregate, and GSH *S*-transferase fractions have only minimal activity of GSH peroxidase when either H_2O_2 or cumene hydroperoxide is used as a substrate. The protein with GSH peroxidase activity has a mol.wt. of 85 000 and its properties are similar to those of human placental GSH peroxidase. The ratios of GSH peroxidase and GSH *S*-transferase activities at various steps during the purification of the 65%-saturated- $(\text{NH}_4)_2\text{SO}_4$ fraction are given in Table 2. The ratio approaches zero at the final step, indicating that purified anionic GSH *S*-transferases have no GSH peroxidase activity. During the

DEAE-cellulose column chromatography this ratio increases severalfold. Until this step, both activities co-purify, and the increase in ratio is probably due to the loss of some GSH S-transferase activity during this step.

The affinity chromatography on GSH linked to epoxy-activated Sepharose 6B results in about 25-fold purification of the anionic GSH S-transferase over the previous step. During the affinity chromatography a substantial amount of enzyme activity is lost, probably because of the high pH (9.6) of the elution buffer. On isoelectrofocusing, anionic GSH S-transferases separate into a major peak along with a very minor peak of enzyme activity (Fig. 2). When both these peaks are pooled separately and subjected individually to re-isoelectrofocusing on a thin-layer bed of Sephadex G-75 by the method described by Srivastava *et al.* (1976), they appear as single peaks corresponding to the pI of the parent fractions. During several isoelectrofocusing experiments the pI values of GSH S-transferase ω (major component) and ψ (minor component) were found to be about 4.6 and 5.4 respectively.

Properties of GSH S-transferase ω

The mol.wt. of GSH S-transferase ω as determined by Sephadex G-150 gel filtration is about 46000. The subunit mol.wt. of about 22500 indicates the enzyme to be a dimer. The molecular weights of various species of GSH S-transferases not only of human tissues, but also of bovine (Awasthi *et al.*, 1980) and rodent (Jakoby, 1978) tissues, are also in the same range. However, during the present studies we observed a significant difference in the subunit size of GSH S-transferase ω and those of cationic species of GSH S-transferase present in human liver. Polyacrylamide-disc-gel electrophoresis in the presence of urea, SDS and β -mercaptoethanol indicated that GSH S-transferase has two subunits of identical size (22500 daltons). On the other hand, the subunit mol.wt. of cationic GSH S-transferases determined by a similar method was about 24500, a value that appears to be in agreement with earlier reported values (Jakoby, 1978) for the subunits of these enzymes. Since the difference in the molecular size of the subunit of GSH S-transferase ω and the cationic GSH S-transferases was relatively small for them to be well-differentiated on urea/SDS/polyacrylamide-disc-gel electrophoresis, we decided to co-electrophorese these enzymes. When a mixture of GSH S-transferase ω and cationic GSH S-transferase was subjected to urea/SDS/polyacrylamide-disc-gel electrophoresis two protein bands corresponding to the mol.wt. of 22500 and 24500 were observed (Fig. 3b).

The amino acid composition of GSH S-trans-

ferase ω is given in Table 3. Except for some similarity in the contents of glutamate, arginine and alanine, the amino acid composition of GSH S-transferase ω appears to be significantly different from those of basic GSH S-transferases (α , β , γ , δ and ϵ) of human liver reported by Kamisaka *et al.* (1975). The differences between the amino acid compositions of GSH S-transferase ω and GSH S-transferase ρ of human erythrocytes (Marcus *et al.*, 1978) are less conspicuous. There are, however, some remarkable differences between the amino acid compositions of these two proteins. Unlike GSH S-transferase ρ , methionine is totally absent from GSH S-transferase ω and the histidine and tyrosine contents of the two proteins are significantly different.

The specific activity of homogeneous GSH S-transferase ω for 1-chloro-2,4-dinitrobenzene was 26 units/mg of protein. No detectable activity was seen when 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 4-nitropyridine *N*-oxide were used as substrates. The apparent K_m and V_{max} for 1-chloro-2,4-dinitrobenzene were 0.83 mM and 360 mol/min per mol of protein respectively. The apparent K_m for GSH was 0.18 mM and the pH optimum of the enzyme was 7.5 when 1-chloro-2,4-dinitrobenzene was used as substrate. The

Table 3. Amino acid composition of human liver GSH S-transferase ω

Each value represents the average of three samples hydrolysed for 24, 48 and 96 h, with the exception of serine and threonine, where the values were extrapolated to zero time by linear-regression analysis. The values for valine and isoleucine are that from the 96 h hydrolysis. Experimental details are given in the text.

	Amino acid composition (no. of residues/molecule of M_r 46 000)
Aspartic acid	45.45 $\pm$ 2.47
Threonine	24.4
Serine	30.3
Glutamic acid	50.37 $\pm$ 2.72
Proline	29.60 $\pm$ 4.42
Glycine	45.11 $\pm$ 2.15
Alanine	33.22 $\pm$ 2.18
Valine	37.3
Isoleucine	14.0
Leucine	48.28 $\pm$ 3.65
Tyrosine	13.25 $\pm$ 1.16
Phenylalanine	14.69 $\pm$ 0.98
Lysine	24.36 $\pm$ 1.17
Histidine	9.73 $\pm$ 1.73
Arginine	20.64 $\pm$ 0.87
Methionine	—

homogeneous enzyme expressed no GSH peroxidase activity whatsoever when either H_2O_2 or cumene hydroperoxide was used as substrate. The energy of activation of the enzyme was found to be 44.52 kJ/mol.

Cationic GSH *S*-transferases

Through a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange chromatography over CM-cellulose and affinity chromatography over GSH bound to epoxy-activated Sepharose 6B we have developed an efficient and quick method for the purification of these enzymes in reasonably high yield (Table 1). During the final step of isoelectric focusing cationic GSH *S*-transferases separated into five peaks in the range pI 8.5–9.5 (Fig. 4). These results, though not identical, are similar to earlier reports (Kamisaka *et al.*, 1975). All the peaks of GSH *S*-transferases obtained during isoelectric focusing were pooled together rather than being pooled separately. Urea/SDS/ β -mercaptoethanol/polyacrylamide-disc-gel electrophoresis reveals the presence of a single band corresponding to a mol.wt. of about 24 500. The specific activity of the pooled cationic GSH *S*-transferase is 14.5 units/mg of protein for 1-chloro-2,4-dinitrobenzene.

As evident from Table 2, cationic GSH *S*-transferases express significant amounts of GSH peroxidase activity. The GSH peroxidase activity of cationic GSH *S*-transferase is displayed in all fractions during the purification. Some decline observed in the GSH peroxidase/GSH *S*-transferase activity ratio (Table 2) is probably due to selective loss of GSH peroxidase activity during isoelectric focusing. During isoelectric focusing, several peaks of GSH peroxidase activity are also observed. Interestingly enough, the peaks of GSH *S*-transferase activity and GSH peroxidase activity are not exactly superimposable. GSH peroxidase activity of cationic GSH *S*-transferase is not expressed towards H_2O_2 or even *t*-butyl hydroperoxide, but is expressed only towards cumene hydroperoxide. The activity towards cumene hydroperoxide was cyanide-insensitive, and was not affected by preincubating the enzyme with Drabkin's reagent [0.03% $\text{K}_3\text{Fe}(\text{CN})_6$ and 0.01% NaCN]. These observations indicate that the GSH peroxidase activity of cationic GSH *S*-transferase is not expressed because of haem or other heterocyclic hydrophobic compounds, which are known to bind GSH *S*-transferase. Also, purified cationic GSH *S*-transferase protein did not exhibit the characteristic u.v.-absorption spectrum of haem.

The antibodies raised against the pooled homogeneous cationic GSH *S*-transferases cross-react with GSH *S*-transferase ω . In a double-immunodiffusion study both anionic and cationic GSH *S*-transferases showed a precipitin line with the

Fig. 5. Immunotitration of GSH *S*-transferase ω (■) and cationic GSH *S*-transferases (○). Details are given in the text.

antibodies raised against cationic GSH *S*-transferases. Similarly, in immunotitration studies, cationic GSH *S*-transferases as well as GSH *S*-transferase ω are precipitated by the antibodies raised against cationic GSH *S*-transferase (Fig. 5). Both GSH *S*-transferase and GSH peroxidase activities of cationic GSH *S*-transferase are precipitated by the antibodies.

Discussion

The present study reveals the presence of two additional GSH *S*-transferases (ω and ψ) in human liver. All the species of GSH *S*-transferases reported hitherto from human liver are cationic proteins, with pI values in the range 7.8–8.8, and appear to have similar genetic origin (Kamisaka *et al.*, 1975). Both GSH *S*-transferases ω and ψ , however, are anionic proteins. Although the amino acid composition of GSH *S*-transferase ω appears to be significantly different from that of basic GSH *S*-transferases from human liver, as reported by Kamisaka *et al.* (1975), the immunogenetic similarity between these proteins may indicate that either the genetic origin of GSH *S*-transferase ω is similar to that of cationic GSH *S*-transferase, or at least there are similar antigenic determinants in these two proteins. However, the exact structural and genetic interrelationship

between these proteins can only be ascertained by further studies such as sequencing of these proteins.

Some of the apparent similarities between the amino acid compositions of GSH S-transferase ω and GSH S-transferase ρ (Marcus *et al.*, 1978) prompted us to investigate if GSH S-transferase ω could arise as a result of the contamination of blood in the liver homogenates used for the isolation of this enzyme. The haemoglobin content of the liver homogenate used for the purification of GSH S-transferase ω was determined to estimate the contamination with erythrocyte proteins. Since haemoglobin, which constitutes the bulk of the erythrocyte haemolysate, was found to be only 0.8 mg/ml in the homogenate and the specific activity of GSH S-transferase ρ in the erythrocyte haemolysate was 0.002 unit/mg (Marcus *et al.*, 1978), the contaminating erythrocyte enzyme could account only for less than 1% of GSH S-transferase ω activity in the liver homogenate.

Unlike cationic GSH S-transferases, GSH S-transferases ω and ψ do not express GSH peroxidase II activity. Since the GSH peroxidase activity of cationic GSH S-transferase is expressed only towards cumene hydroperoxide, and H_2O_2 or t-butyl hydroperoxide are not used as substrates by the enzyme, it appears that the hydroperoxide substrate should have a highly hydrophobic nature. Such an activity may have physiological significance, especially for the protection of hydrophobic membrane lipids from peroxidation. On the other hand narrow substrate specificity of GSH S-transferase ω for its transferase activity and its lack of GSH peroxidase activity seem to limit this enzyme in performing the proposed physiological functions of GSH S-transferases and pose questions about its exact physiological functions.

Cationic GSH S-transferase and GSH peroxidase activities co-purify until the apparent homogeneity of the protein as observed by the appearance of a single protein band on urea/SDS/polyacrylamide-disc-gel electrophoresis. This indicates that both these activities are expressed by same protein. However, the failure of these activities to appear as exactly superimposable peaks during the isoelectric focusing of an apparently homogeneous preparation of cationic GSH S-transferases is puzzling, and does not completely rule out the possibility of these activities being expressed by different proteins having minor differences, arising probably as a result of post-translational modifications. Such a speculation would also explain the lack of GSH peroxidase activity in GSH S-transferase ω . It is possible that the post-translational modification of GSH S-transferases might play a role in the regulation of the two catalytic activities of GSH S-transferases. This contention draws support from our studies with bovine GSH S-transferases

(Saneto *et al.*, 1980). It was observed that homogeneous GSH S-transferase isolated from bovine lens was completely devoid of GSH peroxidase activity, yet the antibodies raised against the lens enzyme not only cross-reacted with bovine liver GSH S-transferases but also precipitated GSH peroxidase activity during immunotitrations (Saneto *et al.*, 1980). Thus it is possible that both the catalytic activities of the GSH S-transferase protein have significant physiological roles, and, depending on the need of the specific tissue, their expression is balanced by post-translational modifications of enzyme protein.

This research was supported in part by National Institutes of Health grants EY 01677, EY 02260 and CA 27967.

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