Interrelationship between cationic and anionic forms of glutathione *S*-transferases of bovine ocular lens

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Since the eye is constantly exposed to potentially damaging chemical compounds present in the atmosphere and vascular system, we investigated the physiological role of glutathione *S*-transferase (GSH *S*-transferase) in detoxification mechanisms operative in the ocular lens. We have purified an anionic and a cationic GSH *S*-transferase from the bovine lens to homogeneity through a combination of gel filtration, ion-exchange and affinity chromatography. The anionic (pI 5.6) and cationic (pI 7.4) *S*-transferases were found to have distinct kinetic parameters (apparent K_m and V_{max} , pH optimum and energy of activation). However, both species were demonstrated to have similar molecular weights and amino acid compositions. Double-immunodiffusion and immunotitration studies showed that both lens *S*-transferases were immunologically similar. The very close similarity in amino acid compositions and immunological properties strongly indicates that these two transferases either originate from the same gene or at least share common antigenic determinants and originate from similar genes. The bovine lens GSH *S*-transferases had no glutathione peroxidase activity with either *t*-butyl hydroperoxide or cumene hydroperoxide as substrate. However, the antibody raised against the homogeneous anionic glutathione *S*-transferase from the bovine lens was found to precipitate both glutathione *S*-transferase and glutathione peroxidase activities out of solution in the supernatant of a crude bovine liver homogenate.

The eye is constantly being exposed to damaging chemical compounds, such as dihalomethanes, azo-dyes and methylcholanthrene, present in the atmosphere and vascular system. However, the toxic effect of these compounds and their metabolites arising from their possible oxygenation by microsomal oxygenases is not known. Glutathione *S*-transferase (EC 2.5.1.18) acts as a system for detoxification and subsequent removal of such agents (Jakoby & Keen, 1977). In every tissue investigated except the human erythrocyte, the glutathione *S*-transferases (GSH *S*-transferases) are present in multiple forms, and different modes of origin have been proposed (Habig *et al.*, 1974; Kamisaka *et al.*, 1975).

The GSH *S*-transferases are a family of proteins having multiple detoxification functions (Habig *et al.*, 1974; Keen *et al.*, 1976; Jakoby & Keen, 1977; Jakoby, 1978). Six of the GSH *S*-transferases from rat liver (Habig *et al.*, 1974; Keen *et al.*, 1976) and

five from the human liver (Kamisaka *et al.*, 1975) have been purified to homogeneity. On the basis of the amino acid compositions and immunological studies, Habig *et al.* (1974) have inferred that rat liver GSH *S*-transferases probably arise from at least three different gene products. On the other hand, all five of the human liver basic GSH *S*-transferases (pI 7.8–8.8) are immunologically cross-reactive, have very similar amino acid compositions and have been suggested to arise by deamidation *in vivo* of a single gene product (Kamisaka *et al.*, 1975). GSH *S*-transferase ρ , present in human erythrocytes (Marcus *et al.*, 1978), has been suggested to be a separate gene product from the liver enzymes.

Prohaska & Ganther (1977) demonstrated that a partially purified rat liver GSH *S*-transferase expressed selenium-independent GSH peroxidase activity (Lawrence & Burk, 1976). This would indicate that GSH *S*-transferase has an additional physiological role of protection against oxidative stress. Since the lens is a long-lived tissue with little protein turnover, it may be a better tissue with which

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

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to study the multiple complement of GSH *S*-transferases and their possible origin.

In the present paper we demonstrate the presence of an anionic GSH *S*-transferase in bovine lens. This enzyme was purified to homogeneity and some of its kinetic, structural and immunological properties were studied.

Materials and methods

Materials

Eyes were obtained from freshly slaughtered cattle, and the lenses with intact capsule were removed from the eye globe and frozen at -30°C until used. *p*-Nitrobenzyl chloride was purchased from Aldrich Chemical Company, Milwaukee, WI, U.S.A. Antibodies developed against rat liver GSH *S*-transferases A, B and E were kindly provided by Dr. W. B. Jakoby of National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A. The sources of other chemical used were same as those reported in the preceding paper (Awasthi *et al.*, 1980a).

Enzyme assay

GSH peroxidase activity was assayed by a modification of the procedure of Awasthi *et al.* (1975), by using two blanks, one without cumene hydroperoxide or *t*-butyl hydroperoxide and the other without enzyme (Awasthi *et al.*, 1980a).

GSH *S*-transferase activity was determined with 1-chloro-2,4-dinitrobenzene as the substrate (Habig *et al.*, 1974). The enzyme assays with other substrates such as 4-nitropyridine *N*-oxide, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrobenzyl chloride were performed by the method described elsewhere (Fjellstedt *et al.*, 1973; Habig *et al.*, 1974), and bilirubin and probenecid binding to the enzyme protein was done by the method of Ketley *et al.* (1975) and Kaplowitz *et al.* (1976). The unit of enzyme activity was defined as $1\mu\text{mol}$ of substrate conjugated or cleaved/min at 25°C for GSH *S*-transferase and at 37°C for GSH peroxidase. Protein was measured by the method of Bradford (1976), with bovine serum albumin as the reference standard.

Purification of glutathione *S*-transferase

Preparation of lens extract. Unless otherwise mentioned, all purification steps were performed at 4°C . Six decapsulated bovine lenses were homogenized in 10mM-potassium phosphate buffer, pH 7.2, containing 1.4mM- β -mercaptoethanol (buffer A) in a Sorvall Omni-Mixer at 8000 rev./min. To avoid heating, the homogenization was performed in three bursts of 20s each, with an interval of 2min between each burst. The homogenate was centrifuged at 10000g for 30min and the super-

natant (70ml) was passed through a Sephadex G-200 column.

Sephadex G-200 gel filtration. The supernatant was applied to a Sephadex G-200 column ($5\text{cm} \times 100\text{cm}$) by upward flow at a rate of 45 ml/h, and 12ml fractions were collected. The column was pre-equilibrated at this flow rate with buffer A containing 100mM- $(\text{NH}_4)_2\text{SO}_4$. GSH *S*-transferase and GSH peroxidase activities were determined in each fraction. Both the enzyme activities segregated at this stage, and fractions containing GSH *S*-transferase activity, which were completely devoid of GSH peroxidase activity, were pooled and subjected to ion-exchange chromatography.

DEAE-cellulose column chromatography. The pooled GSH *S*-transferase fraction from Sephadex G-200 gel filtration, after overnight dialysis against 20vol. of buffer A, was passed through a DEAE-cellulose column ($2.5\text{cm} \times 40\text{cm}$), equilibrated with buffer A, at a flow rate of 18ml/h. At this stage the enzyme separated into fractions. The enzyme activity in the unadsorbed eluate and wash fractions from DEAE-cellulose column chromatography were designated as cationic GSH *S*-transferase and further purified to homogeneity by the procedure previously reported (Awasthi *et al.*, 1980b). The enzyme activity that was adsorbed on the column was designated as anionic GSH *S*-transferase and eluted with 200ml of a linear gradient of 0–100mM-NaCl in the application buffer. The fractions containing enzymic activity were pooled and dialysed against 8vol. of 22mM-potassium phosphate buffer, pH 7.0 (buffer B).

Purification of anionic GSH *S*-transferases

GSH-affinity chromatography. After dialysis, the fraction from DEAE-cellulose chromatography (anionic species) was passed through a GSH affinity column ($0.75\text{cm} \times 10\text{cm}$) equilibrated with buffer B at a flow rate of 6ml/h. GSH was linked to epoxy-activated Sepharose 6B by the procedure of Simons & Vander Jagt (1977). The coupling of GSH to the epoxide was carried out at pH 7.0, so that it could occur through the thiol group and not the amino groups of GSH. The enzyme was adsorbed on the column, and the column was thoroughly washed free of the unadsorbed proteins with 30ml of buffer B. The enzyme was eluted with 50mM-Tris/HCl buffer (pH 9.4 at 4°C) containing 5mM-GSH. The enzyme, which was eluted as a sharp peak of activity, was pooled and dialysed overnight against 20vol. of buffer A.

Purification of cationic GSH *S*-transferase

The unadsorbed enzyme activity from DEAE-cellulose column chromatography was applied to a GSH affinity column ($0.75\text{cm} \times 10\text{cm}$) equilibrated with buffer B. The column was thoroughly washed

free of unadsorbed proteins and eluted with 50 mM-Tris/HCl buffer (pH 9.4 at 4°C) containing 5 mM-GSH. The eluted enzyme was pooled and dialysed with 20 vol. of 10 mM-potassium phosphate buffer, pH 6.0, containing 1.4 mM- β -mercaptoethanol (buffer C). The dialysed enzyme fraction was then applied to a CM-cellulose column (1.5 cm $\times$ 30 cm) equilibrated with buffer C. After thorough washing, the column was eluted with 500 ml of a 0–100 mM-NaCl linear gradient. The enzyme was eluted as a single sharp peak at an NaCl concentration of about 50 mM. The specific activities of all the fractions constituting the enzyme activity peak were identical. The fractions corresponding to the enzyme-activity peak were pooled and stored.

Extraction of GSH S-transferases from cornea

Bovine corneas were dissected from eye globes and immediately frozen until experimentation. The cornea was cut into small pieces, and the tissue was homogenized by grinding in a pestle and mortar in the presence of an abrasive (finely ground Pyrex glass) in 10 mM-potassium phosphate buffer, pH 7.2, containing 1.4 mM- β -mercaptoethanol. The homogenate was centrifuged at 10000 *g* for 30 min, and the supernatant was assayed for GSH *S*-transferase activity and also used in immunodiffusion experiments.

Partial purification of bovine liver GSH S-transferases

A liver sample from a freshly slaughtered cow was cut into small pieces and homogenized in a Sorvall Omni-Mixer at 8000 rev./min as described above for the lens in buffer A to make a 10% (w/v) homogenate. The homogenate was centrifuged at 10000 *g* for 30 min, and the supernatant was applied to a CM-cellulose CM-52 column (1.5 cm $\times$ 30 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 6.8, containing 1.4 mM- β -mercaptoethanol. The column was thoroughly washed to remove unadsorbed proteins, and the adsorbed enzyme was eluted with 600 ml of a linear gradient of 0–100 mM-NaCl in the application buffer. The fractions containing GSH *S*-transferase activity were pooled and used in the immunological studies.

Gel electrophoresis

Disc-gel electrophoresis in 7.5% polyacrylamide gels was performed as described by Davis (1964). The gels were rapidly stained for protein with Coomassie G-250 by the procedure of Reisner *et al.* (1975). The sensitivity of this method was increased by destaining with three washes, 40 min each wash, with 3.5 (v/v) HClO₄ followed by incubation for 20 min with 5% (v/v) acetic acid. SDS/10%-polyacrylamide-disc-gel electrophoresis was performed as described by Laemmli (1970). Gels were

stained with Coomassie Brilliant Blue R 250 and destained by the procedure of Matsudaira & Burgess (1978). Urea/polyacrylamide-disc-gel electrophoresis in 7.5% polyacrylamide containing deionized 8 M-urea was performed by using the Laemmli (1970) system.

Isoelectrofocusing

A portion of purified anionic GSH *S*-transferase (0.1 mg) was dialysed against double-distilled water (four changes of 1 litre each). The enzyme fraction was then subjected to isoelectrofocusing in an LKB model 8100-1 isoelectrofocusing column. Ampholines were used in the pH range 3.5–10 (final concn. 1%, w/v) in a 0–50% sucrose density gradient. After electrofocusing at 1600 V for 18 h, 0.5 ml fractions were collected.

Amino acid analysis

Amino acid analysis of purified GSH *S*-transferase (both cationic and anionic species) was performed with a Beckman automatic model 121M amino acid analyser. Protein samples containing 10 μ g of enzyme were 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, pH 2.2, and 50 μ l samples were applied to the column for analysis. There were no detectable amino sugars on time-course hydrolysis (Marshall & Neuberger, 1972). Tryptophan was determined by hydrolysis in the presence of 200 μ l of mercaptoethanesulphonic acid for 22 h (Benke *et al.*, 1974). The sample was neutralized with 100 μ l of 4 M-NaOH, and 50 μ l portions were applied to the column for analysis.

Immunological techniques

Antibodies against anionic bovine lens GSH *S*-transferase were made in male albino rabbits by intradermal injection of 60 μ g of purified transferase protein emulsified in 1 ml of Freund's complete adjuvant (Vaitukaitis *et al.*, 1971). Then 3 weeks later the rabbits received a booster containing 50 μ g of protein in Freund's complete adjuvant. Double immunodiffusion with anti-(bovine lens GSH *S*-transferase) serum was performed as described by Ouchterlony (1958). Immunotitration studies were performed by incubating a fixed amount of enzyme with different amounts of antiserum raised in rabbits against the anionic species. The reaction mixture, total volume 200 μ l in 10 mM-potassium phosphate buffer, pH 7.0, was incubated overnight at 4°C. Subsequently goat anti-(rabbit immunoglobulin G) serum (50 μ l) was added, and the reaction mixture was incubated overnight again at 4°C, centrifuged at 10000 *g* for 30 min, and the supernatant was used for enzyme activity determination.

Sucrose-density-gradient ultracentrifugation

Ultracentrifugation was performed by the method of Martin & Ames (1961). To determine the molecular weights of the purified enzymes, 6 ml of a linear 10–25% (w/v) sucrose density gradient in 50 mM-potassium phosphate buffer, pH 6.8, was layered with 0.2 ml of either (anionic or cationic) GSH *S*-transferase or protein standards (β -galactosidase, mol.wt. 130 000; bovine serum albumin, mol.wt. 68 000; purified hen's-egg albumin, mol.wt. 45 000; chymotrypsinogen A, mol.wt. 23 900; ribonuclease A, mol.wt. 14 000).

Results

Purification of GSH *S*-transferase

Purification of anionic GSH *S*-transferase from bovine lens is summarized in Table 1. GSH *S*-transferase, which fractionated with a mol.wt. size of 49 000, was eluted from the DEAE-cellulose column at 20–30 mM-NaCl (Fig. 1). The pooled enzyme fractions from GSH-linked Sepharose affinity chromatography showed a single protein band on electrophoresis of an overloaded (60 μ g of protein) polyacrylamide disc gel (Fig. 2a). A densitometer scan of the stained gel for protein showed a single sharp peak (Fig. 2a). SDS/ β -mercaptoethanol/urea/polyacrylamide-disc-gel electrophoresis also showed a single protein band, indicating that the purified enzyme preparation was apparently homogeneous (Fig. 2b). The homogeneous anionic GSH *S*-transferase (specific activity 4.61 units/mg of protein) was purified 105-fold with a yield of 43%. Polyacrylamide-disc-gel electrophoresis of the cationic GSH *S*-transferase or an overloaded (60 μ g) gel also showed a single protein band (Fig. 3).

Separation of GSH peroxidase and GSH *S*-transferase activities

During Sephadex G-200 chromatography, GSH *S*-transferase was eluted in a sharp peak (Fig. 4)

Fig. 1. DEAE-cellulose column chromatography of anionic bovine lens GSH *S*-transferase. Details are given in the text. ●, GSH *S*-transferase activity; ----, NaCl gradient.

Fig. 2. Polyacrylamide-disc-gel electrophoresis of anionic GSH *S*-transferase of bovine lens

Experimental details are given in the text. (a) Anionic GSH *S*-transferase in the system of Davis (1964). Superimposed is a drawing of the densitometer scan of the gel. (b) SDS/ β -mercaptoethanol/urea/polyacrylamide-disc-gel electrophoresis of anionic GSH *S*-transferase.

Table 1. Purification summary of GSH *S*-transferases from bovine lens
Experimental details are given in the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Homogenate	70	5524.40	243.97	0.044	—
Sephadex G-200	465	1274.10	241.80	0.190	99
DEAE-cellulose (adsorbed anionic GSH <i>S</i> -transferase)	178	82.24	9.87	0.121	4
DEAE-cellulose (unadsorbed cationic GSH <i>S</i> -transferase)	130	444.60	169.27	0.381	69
Anionic GSH <i>S</i> -transferase					
DEAE-cellulose	178	82.24	9.87	0.121	100
Affinity chromatography	11	0.91	4.21	4.61	43

with an elution volume corresponding to a mol.wt. of approx. 49 000, whereas GSH peroxidase activity was eluted in the position corresponding to a mol.wt. of approx. 85 000. The purified GSH *S*-transferases had no GSH peroxidase activity towards H_2O_2 , *t*-butyl hydroperoxide or cumene hydroperoxide.

Fig. 3. Polyacrylamide-disc-gel electrophoresis of cationic GSH *S*-transferase of bovine lens. Experimental details are given in the text. Superimposed is a drawing of the densitometer scan of the gel.

Molecular-weight determinations

The molecular mass of homogeneous anionic bovine lens GSH *S*-transferase as estimated on a calibrated column (2.5 cm $\times$ 100 cm) of Sephadex G-100 was approx. 49 000 daltons. On ultracentrifugation, in a sucrose density gradient the homogeneous anionic and cationic bovine lens GSH *S*-transferases were found to migrate together and slightly ahead of hen's-egg ovalbumin (molecular mass of 45 000 daltons). The molecular mass of GSH *S*-transferases was calculated to be about 46 000 daltons.

Subunit structures of anionic and cationic GSH *S*-transferases

The interaction between the subunits of the anionic and cationic enzymes was investigated by using polyacrylamide-disc-gel electrophoresis in the presence of SDS or urea alone. On SDS/urea/ β -mercaptoethanol/polyacrylamide-disc-gel electrophoresis the anionic GSH *S*-transferase dissociated into two subunits of molecular mass 23 500 daltons. The enzyme protein dissociated into subunits in the presence of urea alone or SDS alone. The urea dissociated subunits migrated on polyacrylamide-disc-gel electrophoresis as a single protein band, indicating that the subunits have similar charge and are not held together by disulphide bonds.

Polyacrylamide-disc-gel electrophoresis of the cationic GSH *S*-transferase in 10% polyacrylamide gels containing 0.1% SDS also revealed a single protein band migrating to the same position as did the protein subjected to SDS/urea/ β -mercapto-

Fig. 4. Sephadex G-200 gel filtration of bovine lens GSH *S*-transferase and GSH peroxidase. Details are given in the text. Δ , A_{280} (enzyme protein); O, GSH peroxidase activity; $\square$, GSH *S*-transferase activity.

ethanol/polyacrylamide-gel electrophoresis. The cationic enzyme species in the presence of 8 M-urea also showed a single protein band.

Amino acid composition

The amino acid compositions of both anionic and cationic GSH *S*-transferases are given in Table 2. The partial specific volumes of these enzymes calculated from the amino acid composition by the method of Cohn & Edsall (1943) were $0.732 \text{ cm}^3 \cdot \text{g}^{-1}$ for the anionic and $0.730 \text{ cm}^3 \cdot \text{g}^{-1}$ for the cationic species.

Comparison of different methods used for protein determination

The homogeneous fraction of the anionic GSH *S*-transferase was found to have a protein concentration of 0.083 mg/ml by the method of Bradford (1976), and 0.027 mg/ml from the amino acid analysis. Similarly, the homogeneous fraction of the cationic GSH *S*-transferase was found to have a protein concentration of 0.049 mg/ml by the method of Bradford (1976), and 0.011 mg/ml from the

amino acid analysis. Bovine serum albumin was used as the standard for the Bradford (1976) method of protein determination.

Isoelectric pH and pH optimum

When the anionic GSH *S*-transferase was subjected to isoelectric focusing, a single sharp peak of activity was observed. The peak fraction had a pI of 5.66.

The enzyme reaction determined after compensation for the non-enzymic conjugation of GSH with 1-chloro-2,4-dinitrobenzene exhibited a broad pH optimum between 6 and 7.

Substrate specificity

The apparent K_m and V_{max} for the anionic GSH *S*-transferase determined by least-squares regression analysis of four separate experiments for 1-chloro-2,4-dinitrobenzene with GSH as the second substrate were $0.158 \pm 0.021 \text{ mM}$ and $439.7 \pm 24.4 \text{ mol/min per mol of protein respectively (means} \pm \text{s.d.)}$. The apparent K_m and V_{max} of anionic GSH *S*-transferase for GSH with 1-chloro-2,4-dinitrobenzene as the second substrate were 0.122 mM and $330.5 \text{ mol/min per mol of protein respectively}$. Under identical conditions the apparent K_m and V_{max} of the cationic GSH *S*-transferase for GSH were 0.121 mM and $197.7 \text{ mol/min per mol of protein respectively}$.

The effect of temperature on GSH *S*-transferase was studied by determining the enzyme activity at various temperatures with 1-chloro-2,4-dinitrobenzene as substrate. The energy of activation of the anionic lens enzyme was found to be approx. 53.1 kJ/mol .

Bovine lens anionic GSH *S*-transferase was found to have a specific activity of 0.06 unit/mg of protein towards *p*-nitrobenzyl chloride, and that towards 1-chloro-2,4-dinitrobenzene was 4.61 units/mg of protein. The transferase was found to have no detectable activity towards either 1,2-epoxy-3-(*p*-nitrophenoxy)propane or 4-nitropyridine *N*-oxide.

The anionic GSH *S*-transferase required GSH as the second substrate. Cysteine, β -mercaptoethanol and dithiothreitol were unable to replace GSH in the standard assay with 1-chloro-2,4-dinitrobenzene.

Anionic GSH *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene was found to be partially inhibited by the presence of probenecid and bilirubin. At substrate concentrations of 0.16 mM 1-chloro-2,4-dinitrobenzene and 1.0 mM -GSH, $0.25 \mu\text{M}$ bilirubin and 1.75 mM -probenecid inhibited conjugation of 1-chloro-2,4-dinitrobenzene to GSH by 51% and 39% respectively.

Immunological parameters

Immunotitration and Ouchterlony immunodiffusion experiments were performed with antisera

Table 2. Amino acid compositions of anionic and cationic bovine lens GSH *S*-transferases

All the values, except those marked by symbols, are means of 24, 48 and 96 h of hydrolysis in 5.7 M-HCl, and expressed as mol of residue/mol of GSH *S*-transferase (mean $\pm$ s.d., $n = 3$). Experimental details are given in the text.

Amino acid	Amino acid composition (mol of residue/mol of enzyme)	
	Anionic enzyme	Cationic enzyme
Aspartic acid	47.67 ± 1.22	49.95 ± 0.39
Threonine*	11.42	14.66
Serine*	14.32	20.92
Proline	25.43 ± 1.37	28.59 ± 2.09
Glycine	38.54 ± 1.62	30.95 ± 1.36
Alanine	20.77 ± 0.49	23.39 ± 0.51
Cysteine	13.43	3.53
(half-cystine)*		
Valine†	15.63	16.36
Methionine	13.60 ± 0.75	9.46 ± 0.36
Isoleucine†	24.13	25.66
Leucine	50.50 ± 1.02	52.41 ± 0.47
Glutamic acid	52.49 ± 0.92	42.99 ± 0.59
Tyrosine	17.49 ± 0.68	17.42 ± 0.14
Phenylalanine	24.80 ± 0.63	26.04 ± 0.91
Lysine	31.84 ± 0.56	35.05 ± 0.52
Histidine	7.80 ± 0.44	9.20 ± 0.38
Arginine	24.09 ± 0.56	27.59 ± 0.45
Tryptophan‡	7.32	—

* Value obtained by extrapolation to zero time by least-squares linear-regression analysis.

† Value obtained for the 96 h hydrolysis.

‡ Value obtained by 22 h hydrolysis in mercaptoethanesulphonic acid.

raised against homogeneous bovine lens anionic GSH *S*-transferase. Immunotitration of anionic and cationic enzymes showed that both activities are precipitated out of solution almost identically, to a complete loss of enzyme activity (Fig. 5). Double-antiserum immunotitration was performed with partially purified bovine liver fractions. The partially purified liver fractions were pooled into two different groups of GSH *S*-transferase activity on the basis of CM-cellulose chromatography, namely the unadsorbed enzyme activity and the eluted fractions of

enzyme activity that adsorbed on the column. The antisera precipitated only the unadsorbed enzyme but not the enzyme that was adsorbed on the CM-cellulose column. In a separate experiment with the bovine liver homogenate supernatant obtained by centrifugation at 10000*g* for 30 min, the antiserum partially precipitated both the GSH *S*-transferase and the GSH peroxidase activities (determined with *t*-butyl hydroperoxide or cumene hydroperoxide) out of solution (Fig. 6). The results of double-immunodiffusion experiments are given in Fig. 7. Antisera raised against homogeneous anionic GSH-*S*-transferase from bovine lens cross-reacted with cationic GSH *S*-transferase of lens and anionic GSH *S*-transferase of liver. However, the antisera did not cross-react with cornea GSH *S*-transferase and cationic liver GSH *S*-transferase.

Fig. 5. Immunotitration of anionic and cationic bovine lens GSH *S*-transferases by antiserum raised against homogeneous anionic bovine lens GSH *S*-transferase. Details are given in the text. ■, Cationic GSH *S*-transferase activity in the presence of normal serum; ●, anionic GSH *S*-transferase activity in the presence of normal serum; ▲, cationic GSH *S*-transferase activity in the presence of antiserum; ○, anionic GSH *S*-transferase activity in the presence of antiserum.

Fig. 7. Immunotitration of bovine liver GSH *S*-transferase and GSH peroxidase. Details are given in the text. ●, GSH *S*-transferase activity; ○, GSH peroxidase activity with cumene hydroperoxide as substrate; △, GSH peroxidase activity with *t*-butyl hydroperoxide as substrate.

Fig. 6. Double-immunodiffusion studies of bovine liver and ocular GSH *S*-transferases

Details are given in the text. The centre well of both immunodiffusion plates contains antiserum raised against homogeneous anionic bovine lens GSH *S*-transferase. In (a), wells 1 and 4 contained homogeneous anionic bovine lens GSH *S*-transferase, well 2 contained homogeneous cationic bovine lens transferase, well 3 contained bovine cornea extract and well 5 contained bovine liver extract. In (b), wells 1 and 2 contained homogeneous anionic bovine lens GSH *S*-transferase, well 3 contained anionic bovine liver GSH *S*-transferase, well 4 contained homogeneous cationic bovine lens GSH *S*-transferase and well 5 contained cationic bovine liver GSH *S*-transferase extract.

During the present study the Bradford (1976) protein assay was used to determine protein content with bovine serum albumin as the reference standard. The estimate of protein content of the homogeneous enzyme, both anionic and cationic species, by amino acid composition was considerably lower than that estimated by the Bradford (1976) assay. This suggests that Bradford (1976) assay method may give an apparently higher protein value if the sample contains hydrophobic protein(s) and albumin is used as reference standard.

Discussion

The highly anionic GSH *S*-transferase (pI 5.6) purified to homogeneity from the bovine lens was found to have significant differences in kinetic properties such as pH optimum, energy of activation, apparent K_m and V_{max} , for 1-chloro-2,4-dinitrobenzene when compared with the more cationic bovine lens GSH *S*-transferases (pI 7.4). However, the structural properties such as amino acid composition, molecular weight and subunit structure were comparable (Table 3).

Since both anionic and cationic GSH *S*-transferases have marked similarity in amino acid compositions (Table 2), and antiserum raised against the anionic species precipitated both *S*-transferase activities equally and completely out of solution (Fig. 5), it may be postulated that the two bovine lens GSH *S*-transferases arise from a post-translational modification of a single gene product. Furthermore, both anionic and cationic species were found to be composed of subunits having an identical molecular mass, about 23 500 daltons. In the presence of 8 M-urea the subunits of both anionic and cationic GSH *S*-transferases migrated as one protein band. These studies also indicate that the two GSH *S*-transferases could originate from a modification of a single gene product. The present results, however, do not rule out the possibility that these proteins

arise from similar yet different genes and only share some of the common antigenic determinants.

The lens is a long-lived tissue with an extremely slow protein turnover, and synthesis of RNA and protein occur in mainly the more superficial cells; therefore the newly synthesized protein represents only a minor fraction of the total protein (Hanna, 1965; Young & Fulhous, 1966). As a result, the nucleus of the adult lens contains many of the proteins formed during foetal life (van Kleef *et al.*, 1975). van Kleef *et al.* (1975) have demonstrated that bovine lens α -crystallin subunits undergo post-translational modifications to produce multiple species, and that the amount of each modified α -crystallin increased with aging. Thus, if the cationic and anionic GSH *S*-transferases arise from a single gene product, it is possible that the anionic species representing only 5% of total GSH *S*-transferase is the primary gene product and the cationic species representing about 95% of the enzyme originates as a result of post-translational modification(s).

Prohaska & Ganther (1977) have shown that a partially purified fraction of rat liver GSH *S*-transferase (molecular mass 45 000 daltons), having no selenium, displayed GSH peroxidase activity towards the organic hydroperoxides, but not towards H_2O_2 . During the present study we have demonstrated that both anionic and cationic bovine lens GSH *S*-transferases do not express GSH peroxidase activity with H_2O_2 , cumene hydroperoxide or *t*-butyl hydroperoxide as substrate. However, our results indicate that bovine liver GSH *S*-transferase expresses GSH peroxidase activity, because during double immunotitration the antisera raised against bovine lens anionic GSH *S*-transferase not only precipitated GSH *S*-transferase out of the supernatant from a crude bovine liver homogenate fraction but also precipitated GSH peroxidase activity (Fig. 5). Our studies with purified GSH *S*-transferase from human liver also indicated that this enzyme has GSH peroxidase activity

Table 3. Comparison of kinetic and structural properties for anionic and cationic bovine lens GSH *S*-transferases
 V_{max} values are expressed in mol/min per mol of protein, and K_m values in mM.

Property	Anionic GSH <i>S</i> -transferase	Cationic GSH <i>S</i> -transferase
K_m (1-chloro-2,4-dinitrobenzene)	0.16	0.44
V_{max} (1-chloro-2,4-dinitrobenzene)	440	730
K_m (GSH)	0.12	0.12
V_{max} (GSH)	320	197
pH optimum	6.0–7.0	7.0
Energy of activation (kJ/mol)	53.1	46.7
Partial specific volume	0.732	0.730
Molecular weight	49 000	49 000
Subunit molecular weight	23 500	23 000

(Awasthi *et al.*, 1980a). Therefore these studies indicate that the post-translational modifications in the GSH S-transferase that occur in the liver may be different from that occurring in the lens. Such modifications provide the liver with additional GSH peroxidase activity, which may be important in the defence of the liver against oxidative damage.

Both homogeneous anionic and cationic bovine lens GSH S-transferases have similar molecular weights and subunit structures to those reported for homogeneous rat and human hepatic and human erythrocyte GSH S-transferases. Although there is a similarity in amino acid compositions between the human liver and bovine lens GSH S-transferases, antisera raised against anionic bovine lens GSH S-transferase do not cross-react with the human liver GSH S-transferases. None of the antibodies to rat liver preparations was cross-reactive towards the human liver (Jakoby, 1978) or bovine lens GSH S-transferases. Interestingly, the kinetic properties of the bovine lens GSH S-transferases appear to be dissimilar to those of the rat and human liver enzymes. The bovine lens anionic and cationic GSH S-transferases have pH optima of 6.0–7.0 and 7.0 respectively, whereas the pH optimum for the human and rat liver GSH S-transferases is 7.5 (Boyland & Chasseaud, 1967; Kamisaka *et al.*, 1975). The apparent K_m of the lens cationic enzyme for the substrate 1-chloro-2,4-dinitrobenzene appears to be similar to that of the human enzymes. However, the anionic enzyme had significantly lower K_m than that reported for hepatic GSH S-transferase from other species. Substrate specificity is also narrower in the bovine lens GSH S-transferases. The anionic species has activity towards *p*-nitrobenzyl chloride, whereas the cationic species does not. Both species had no activity towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 4-nitropyridine *N*-oxide. Therefore the lens GSH S-transferases, which seem structurally similar in certain respects to the other homogeneous mammalian transferases, have certain distinctive kinetic properties.

Interestingly, the cornea GSH S-transferase appears to be genetically distinct from the lens because the antiserum raised against the lens enzyme does not precipitate the cornea GSH S-transferase. The biological role of different forms of GSH S-transferase found in different ocular tissues and their role in the biotransformation of xenobiotics remains to be studied.

GSH S-transferase present in cornea and lens can conjugate xenobiotics and other electrophilic substances to GSH, which leads to the formation of mercapturic acid (Barnes *et al.*, 1959; Bray *et al.*, 1959). All of the enzymes, including γ -glutamyl transpeptidase known to be involved in the mercapturic acid-biosynthetic pathway, are present in the lens and cornea (Miller *et al.*, 1976). It is

noteworthy that GSH S-transferase constitutes 1.5% of the total soluble protein in the lens, and the lens has almost the highest content of GSH found in any tissue. Therefore GSH probably has the additional function of detoxification in the lens.

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