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Glutathione *S*-transferases of the bovine retina

Evidence that glutathione peroxidase activity is the result of glutathione *S*-transferase

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We have purified two isoenzymes of glutathione *S*-transferase from bovine retina to apparent homogeneity through a combination of gel-filtration chromatography, affinity chromatography and isoelectric focusing. The more anionic (pI = 6.34) and less anionic (pI = 6.87) isoenzymes were comparable with respect to kinetic and structural parameters. The K_m for both substrates, reduced glutathione and 1-chloro-2,4-dinitrobenzene, bilirubin inhibition of glutathione conjugation to 1-chloro-2,4-dinitrobenzene, 1-chloro-2,4-dinitrobenzene inactivation of enzyme activity and molecular weight were similar. However, pH optimum and energy of activation were found to differ considerably. Retina was found to have no selenium-dependent glutathione peroxidase activity. The total glutathione peroxidase activity fractionated with the transferases in the gel-filtration range of mol.wt. 49000 and expressed activity with only organic hydroperoxides as substrate. Only the more anionic isoenzyme expressed both transferase and peroxidase activity.

Glutathione *S*-transferases (EC 2.5.1.18), besides conjugating GSH with xenobiotics, have an additional role of reducing lipid peroxides, i.e. they express selenium-independent glutathione peroxidase activity (Lawrence & Burk, 1976; Prohaska & Ganther, 1977). However, all the forms of glutathione *S*-transferases found in different tissues do not have selenium-independent glutathione peroxidase activity (Saneto *et al.*, 1980; Awasthi *et al.*, 1980). We have recently demonstrated that bovine lens glutathione *S*-transferases did not express glutathione peroxidase activity (selenium-independent), whereas an immunologically similar transferase species in the bovine liver expressed peroxidase activity. To investigate whether the dual enzymic activities of the transferase were limited to the liver, we studied the properties of this enzyme in another tissue exposed to extensive oxidative stress, the retina. This tissue is high in polyunsaturated fatty acids (Forrest & Futterman, 1972), consumes large amounts of oxygen via the abundant mitochondria present and is exposed to large amounts of photodynamic oxidation as a result of photosensitive retinal pigments (Kagan *et al.*, 1973).

We have recently demonstrated the presence of all the enzymes of the mercapturic acid pathway of detoxification in the retina (Saneto, 1981), suggesting retinal glutathione *S*-transferase plays a role in the detoxification of xenobiotics. The multiple detoxification functions of the liver and lens glutathione *S*-transferase have been extensively studied (Habig *et al.*, 1974; Keen *et al.*, 1976; Jakoby, 1978; Awasthi *et al.*, 1980; Saneto *et al.*, 1980). In addition to a detoxification function with exogenous chemicals, these proteins have been shown to protect cellular integrity from endogenous oxidative stress by expressing selenium-independent glutathione peroxidase activity with organic hydroperoxides (Prohaska & Ganther, 1977; Awasthi *et al.*, 1980). However, not all glutathione *S*-transferases express this latter function (Saneto *et al.*, 1980; Awasthi *et al.*, 1980). We have investigated whether the glutathione *S*-transferases found in the retina expressed these heretofore described multiple detoxification functions, as found in the liver. The multiple isoenzymes of glutathione *S*-transferase in the retina were found to have detoxification functions analogous to the liver transferases.

Abbreviations used: GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; SDS, sodium dodecyl sulphate.

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Materials and methods

Materials

Eyes were obtained from freshly slaughtered cattle and retinae were carefully dissected, separated and frozen at −30°C until used. Sephadex G-150, epoxy-activated Sepharose 6B, GSH, NADPH, glutathione reductase (yeast type III), CDNB, bilirubin and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ampholines were obtained from LKB Produktor, Bromma, Sweden. Cumene hydroperoxide and t-butyl hydroperoxide were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *p*-Nitrobenzyl chloride was 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 Institutes of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A. Goat antisera against rabbit γ-globulins were provided by Dr. Philip Rayford, Surgical Biochemistry Laboratory, University of Texas Medical Branch, Galveston, TX, U.S.A.

Methods

Enzyme assay. Glutathione *S*-transferase activity was determined by measuring the conjugation of GSH with CDNB as described by Habig *et al.* (1974). The 1ml assay mixture contained 1mM-CDNB, 1mM-GSH and 0.1M-potassium phosphate buffer, pH6.5; the rate of increase of optical absorbance as measured by using a Gilford recording spectrophotometer model 250 was linear for 5–10min at 25°C, after the addition of appropriate amounts of enzyme. The enzyme activity with the other substrates *p*-nitrobenzyl chloride, 4-nitropyridine-*N*-oxide and 1,2-epoxy-3-(*p*-nitrophenoxy)propane were also measured (Pabst *et al.*, 1973; Fjellstedt *et al.*, 1973; Habig *et al.*, 1974). The effect of bilirubin on the conjugation of GSH with CDNB was estimated by the method of Ketley *et al.* (1975). The covalent binding of CDNB to the enzymes in the absence of GSH was estimated by the method of Jakoby (1978). Glutathione peroxidase activity (selenium-independent and selenium-dependent) was measured by a modification of the procedure of Awasthi *et al.* (1975) by using two blanks, one without t-butyl hydroperoxide, cumene hydroperoxide or hydrogen peroxide and the other without enzyme (Awasthi *et al.*, 1980).

One unit of enzyme activity was defined as 1μmol of substrate conjugated or cleaved per min at 25°C for glutathione *S*-transferase and at 37°C for glutathione peroxidase. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the reference standard.

Purification of glutathione S-transferase. Preparation of retina extract. All purification steps were performed at 4°C. In a typical purification experiment, 15 retinae were homogenized by sonication in 10mM-potassium phosphate buffer, pH7.2, containing 1mM-dithiothreitol (buffer A). The sonication was carried out in three bursts of 15s at 40W with intervals of 15s between each burst. The homogenate was centrifuged at 10000*g* for 30min and the supernatant (30ml) was passed through a Sephadex G-150 column.

Sephadex G-150 gel filtration. The supernatant was applied to a Sephadex G-150 column (2.5cm × 100cm) pre-equilibrated with buffer A with an ascending flow rate of 26ml/h and 5ml fractions were collected. The pooled fractions containing glutathione *S*-transferase activity were dialysed against 10vol. of 22mM-potassium phosphate buffer, pH7.0, containing 1mM-dithiothreitol (buffer B).

Glutathione-affinity chromatography. The dialysed fraction was subjected to glutathione-affinity chromatography. The glutathione-affinity column (0.75cm × 10cm) was equilibrated with buffer B at a flow rate of 6ml/h. GSH was linked to epoxy-activated Sepharose 6B by the procedure of Simons & Jagt (1977). The coupling of GSH to the epoxide was carried out at pH7.0, so that coupling would 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 unadsorbed proteins with 30ml of buffer B. The enzyme was eluted with 50mM-Tris/HCl buffer (pH9.4, at 4°C). The enzyme was eluted in a sharp peak and those fractions containing transferase activity were pooled and dialysed against 100vol. of 1mM-potassium phosphate buffer, pH7.0, containing 1mM-dithiothreitol.

Preparative isoelectric focusing. The dialysed fraction from affinity chromatography was subjected to isoelectric focusing in an LKB model 8100-1 isoelectric focusing 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 1600V for 18h, 0.5ml fractions were collected, assayed for enzymic activity and pH was determined at 4°C.

Glutathione peroxidase activity of glutathione S-transferase. During the purification of glutathione *S*-transferase, glutathione peroxidase activity was measured at each step of purification by using H_2O_2, cumene hydroperoxide and t-butyl hydroperoxide. The peroxidase activity co-eluted with the transferase activity at each step of purification.

Gel electrophoresis. Disc-gel electrophoresis on 7.5% polyacrylamide gels was performed by the method of Davis (1964). The gels were rapidly stained for protein by reaction with Coomassie Blue G-250 by the procedure of Reisner *et al.* (1975). The

sensitivity of this method was increased by the rapid destaining procedure as described previously (Saneto *et al.*, 1980). SDS/urea/β-mercaptoethanol polyacrylamide disc-gel electrophoresis was carried out in 10% polyacrylamide gels by method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 and destained by the procedure of Matsudaira & Burgess (1978).

Analytical isoelectric focusing. A portion of purified glutathione S-transferases (1 unit of enzymic activity) was dialysed against double-distilled water (four changes of 1 litre each). The enzyme fraction was then subjected to isoelectric focusing as described above.

Immunological techniques. Antibodies against bovine lens glutathione S-transferase were produced in rabbits as described previously (Saneto *et al.*, 1980). Double immunodiffusion using anti-(bovine lens glutathione S-transferase) antiserum was carried out as described by Ouchterlony (1958). Immunotitration studies were performed by incubation of a fixed amount of the retina transferases with different amounts of antiserum. The reaction mixture (total volume 200 μl, in 10 mM-potassium phosphate buffer, pH 7.0) was incubated overnight at 4°C. Subsequently goat anti-(rabbit-immunoglobulin G) antiserum (50 μl) was added and the reaction mixture was incubated overnight at 4°C. The reaction mixture was subsequently centrifuged at 10000 *g* for 30 min, and the supernatant was assayed for glutathione S-transferase and glutathione peroxidase activity.

Results

Purification of glutathione S-transferases

Purification of more anionic (pI = 6.34) and less anionic (pI = 6.87) glutathione S-transferase from bovine retina is summarized in Table 1. Glutathione S-transferase during gel filtration on a Sephadex G-150 column fractionated in a single peak with a molecular weight of approx. 49000. Subsequently, the pooled enzyme fractions from glutathione-linked Sepharose affinity chromatography were subjected to isoelectric focusing and found to focus into two distinct fractions. Each of these two fractions was pooled separately and labelled as more anionic and less anionic glutathione S-transferase. Both fractions migrated as a single protein band on electrophoresis in polyacrylamide disc gels. Both these glutathione S-transferases when subjected to SDS/urea/β-mercaptoethanol/polyacrylamide-disc-gel electrophoresis migrated as a single protein band indicating that the purified enzyme preparations were apparently homogeneous by these criteria. The more anionic and less anionic retina transferases had specific activities of 12.72 and 15.47 units/mg of protein respectively.

Glutathione peroxidase activity of glutathione S-transferase

No selenium-dependent glutathione peroxidase activity, as determined by using H_2O_2 (Prohaska & Ganther, 1977), was found in the retina. However, selenium-independent glutathione peroxidase was found in the retina crude homogenate and it co-purified with the glutathione S-transferase. During Sephadex G-150 gel filtration, the glutathione S-transferase and glutathione peroxidase (selenium-independent) activities co-eluted at a molecular weight of approx. 49000 (Fig. 1). The apparently homogeneous more anionic species expressed selenium-independent glutathione peroxidase activity, whereas the less anionic glutathione S-transferase had no glutathione peroxidase activity.

Molecular weight, isoelectric pH and pH optimum

The molecular mass of bovine retina glutathione S-transferases was estimated to be 49000 Da on a

Table 1. *Purification summary of glutathione S-transferases from bovine retina*
Abbreviations used: GSH S-Tr, glutathione S-transferase; GSH-Px, glutathione peroxidase.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)		Specific activity (units/mg of protein)		Yield (%)	
			GSH S-Tr	GSH-Px	GSH S-Tr	GSH-Px	GSH S-Tr	GSH-Px
Homogenate	22.5	40.28	10.58	11.24	0.47	0.52	100	100
Sephadex G-150	112	16.69	9.48	3.47	0.57	0.21	90	31
Affinity chromatography	24	0.26	6.95	*	24.69		66	
Isoelectric focusing								
1. More anionic (pI = 6.34)	23	0.07	1.14	0.12	12.72	1.17	11	
2. Less anionic (pI = 6.87)	23	0.09	1.26		15.47		12	

* After affinity chromatography the activity of selenium-independent glutathione peroxidase could not be accurately determined because dialysis resulted in a total loss of activity and high pH (9.4) interfered in the assay due to non-enzymic oxidation of GSH.

calibrated column (2.5 cm × 100 cm) of Sephadex G-150. Similar results were obtained when homogeneous preparations of both these glutathione *S*-transferases were subjected to Sephadex G-150 gel filtration.

When purified glutathione *S*-transferases were subjected to isoelectric focusing, each isoenzyme focused in a sharp band of activity. The more anionic transferase had a pI of 6.34 and the less anionic transferase had a pI of 6.87. The pI values of the homogeneous transferases were found to correspond to the pI values obtained in the preparative isoelectric-focusing step in the purification procedure.

The pH optimum for enzymic activity, after compensation for non-enzymic conjugation of GSH with CDNB, exhibited a sharp peak of activity at pH 6.5 for the more anionic transferase and a broad peak of activity between pH 6.5 and 7.5 for the less anionic transferase.

Substrate specificity and other kinetic parameters

The apparent K_m and $V_{max.}$ values for the more anionic glutathione *S*-transferase determined by least-squares regression analysis of three separate experiments for CDNB, with GSH as the second substrate, were 1.41 mM and 1105 mol/min per mol of protein (coefficient of determination = 0.97) respectively. The apparent K_m and $V_{max.}$ values for GSH, with CDNB as the second substrate, were 0.17 mM and 593 mol/min per mol of protein ($n = 3$; coefficient of determination = 0.99) respectively.

The less anionic transferase was demonstrated to have an apparent K_m and $V_{max.}$ for CDNB with GSH as the second substrate, as determined by least-squares regression analysis of three separate experiments, of 1.80 mM and 1822 mol/min per mol of protein (coefficient of determination = 1.00) respectively. The apparent K_m and $V_{max.}$ values for GSH, with CDNB as the second substrate, determined in three separate experiments by least-squares regression analysis, were 0.16 mM and 8.27 mol/min per mol of protein (coefficient of determination of 0.96) respectively.

The effect of temperature on the conjugation of GSH with CDNB was studied by using homogeneous glutathione *S*-transferases. The more anionic and less anionic transferases were found to have an energy of activation of 27.1 and 46.2 kJ/mol respectively.

Both the transferases required GSH as the second substrate. Cysteine, β-mercaptoethanol and dithiothreitol were not able to substitute for glutathione in the standard assay with CDNB.

p-Nitrobenzyl chloride, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 4-nitropyridine *N*-oxide were used to determine the degree of substrate specificity. The more anionic transferase having a specific activity of 12.72 units/mg of protein with CDNB, had a specific activity with *p*-nitrobenzyl chloride of 3.02 units/mg of protein. No activity was observed with the other two substrates. The less anionic transferase was found to have no observable activity with any of these three additional substrates tested.

Binding of bilirubin with glutathione S-transferase

Binding of glutathione *S*-transferase by bilirubin was determined by bilirubin inhibition of glutathione conjugated to CDNB (Kamisaka *et al.*, 1975). At substrate concentrations of the approximate K_m value for CDNB, the more anionic retina transferase activity was inhibited by about 30% and the less anionic transferase activity was inhibited by about 25% when 2.5 μM-bilirubin was added to the standard reaction mixture. Bilirubin was demonstrated to be a competitive inhibitor with respect to CDNB, in the conjugation with glutathione by the more anionic transferase. The nature of inhibition by bilirubin was not studied for the cationic transferase because of insufficient material.

Covalent binding by CDNB

Covalent binding of CDNB by the retina transferase was studied by measuring the inactivation of enzymic activity after incubation with this substrate without GSH in the reaction mixture (Pabst *et al.*, 1974). Enzymic activity was subsequently measured by the addition of GSH to the reaction mixture. Adding excess substrates CDNB and GSH did not change the rate of this reaction.

Immunological parameters

Immunotitration and double-immunodiffusion studies were performed by using antisera raised against homogeneous bovine lens anionic glutathione *S*-transferase (Saneto *et al.*, 1980). The antisera did not cross-react with either of the glutathione *S*-transferases purified from the retina.

Discussion

The apparently homogeneous more anionic and less anionic glutathione *S*-transferases of the retina were found to be similar in kinetic and structural parameters (Table 2). Both retina transferases were demonstrated to be covalently bound by the substrate CDNB when GSH was absent and also bind bilirubin. Although the range of the substrates these transferases conjugated to GSH was found to be narrower compared with the liver transferases, both retina transferases conjugated GSH to CDNB, and covalently bound CDNB in the absence of GSH.

Table 2. *Comparison of kinetic and structural properties of anionic and cationic bovine retina glutathione S-transferase*

Property	More anionic transferase	Less anionic transferase
K_m (mM) (CDNB)	1.41	1.80
$V_{max.}$ (mol/min per mol of protein) (CDNB)	1105	1822
K_m (mM) (GSH)	0.17	0.16
$V_{max.}$ (mol/min per mol of protein) (GSH)	593	827
pH optimum	6.5	6.5–7.5
Energy of activation (kJ)	27.1	46.2
pI	6.34	6.87
Molecular weight	49000	49000

This indicated that the retina glutathione *S*-transferases are functionally identical with their counterpart transferases found in the liver.

We have demonstrated that each ocular tissue has different isoenzymes of glutathione *S*-transferase. Although the retina transferases expressed the multiple detoxification mechanism described for the lens transferases, the retina transferases were found to differ kinetically and immunologically from the transferases found in the lens. We have previously demonstrated the lens transferases, anionic and cationic, differed considerably from one another in kinetic parameters and isoelectric pH, yet were similar in molecular weight, subunit size and immunological parameters (Saneto *et al.*, 1980). In contrast with the lens transferases, the retina isoenzymes were very similar to one another in kinetic parameters except in energy of activation and pH optimum for enzyme activity. The retina isoenzymes were also demonstrated to be immunologically distinct from the lens isoenzymes. These data indicate that although the basic detoxification functions of glutathione *S*-transferase are similar in both retina and lens, the isoenzymes described from these two tissues are immunologically distinct. In addition, the substrate specificity of transferases for the lens and retina differ considerably. This difference may lead to difference in the efficiency of detoxification of different xenobiotics by these tissues.

Since the retina does not have a selenium-dependent glutathione peroxidase and this tissue is constantly exposed to oxidants such as H_2O_2 and lipid peroxides, the glutathione peroxidase activity expressed by glutathione *S*-transferase appears to be of paramount importance in the retina.

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