Biochemical Pharmacology, Vol. 32, No. 12, pp. 1843–1850, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

STRUCTURAL, FUNCTIONAL AND HYBRIDIZATION STUDIES OF THE GLUTATHIONE *S*-TRANSFERASES OF RAT LIVER

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(*Received* 6 *July* 1982; *accepted* 10 *December* 1982)

Abstract—We have purified five forms of glutathione *S*-transferase from rat liver. One form was the glutathione *S*-transferase B (ligandin), which is composed of two non-identical subunits with molecular weights of 22,000 (Ya) and 25,000 (Yc). Two of the other transferases were Ya and Yc homodimers. The other two transferases were also homodimers, but their subunit, Yb, had a molecular weight of 24,000. The three proteins containing either Ya or Yc subunits had similar substrate specificities, and all three contained peroxidase activity. The greatest peroxidase activity was present in proteins containing the Yc subunit. Enzymes composed of Yb subunits had minimal peroxidase activity in addition to different substrate specificities. The Ya and Yc containing enzymes bound the ligands bilirubin and indocyanine green with high affinity ($K_D < 5\ \mu M$), although the K_D values of the YcYc protein were consistently 4- to 12-fold greater than those of the other two transferases. Studies were performed to define the origins of the various isozymes. There was no evidence for conversion of Yc to either Ya or Yb during storage or under conditions favorable to proteolysis. Hybridization studies were performed under denaturing conditions (6 M guanidine–HCl), and a YaYc hybrid was formed from the YaYa and YcYc proteins. In addition, both YaYa and YcYc hybrids were formed from transferase B. The hybrids were functionally similar to the proteins isolated originally from the liver. Attempts to form a YaYb hybrid from the YbYb and YaYa transferases were unsuccessful. This result is consistent with the lack of this enzyme form in the liver. Glutathione *S*-transferase B and the Ya and Yc homodimers appeared to be hybrids of common subunits. These three transferases had very similar functional and structural characteristics and differed from the transferases that are composed of Yb subunits.

The glutathione *S*-transferases (EC 2.5.1.18) are a family of enzymes that catalyze the conjugation of electrophilic compounds with glutathione [1]. One form of these transferases is known as ligandin because it binds a variety of non-polar and amphipathic molecules [2]. Recent studies suggest that ligandin and glutathione *S*-transferase B are the same protein [3], but this is not agreed to by all investigators [4].

Glutathione *S*-transferase B is composed of two subunits, referred to as Ya and Yc. These differ slightly in molecular weight, the M_r being, respectively, 22,000 and 25,000 [5–7]. Some investigators have ascribed certain of the unique binding functions of glutathione *S*-transferase B to one of its subunits [8, 9]. Also present in rat liver are two transferases that are homodimers of subunits that are of the same molecular weight as the Ya and Yc subunits of glutathione *S*-transferase B. The YcYc homodimer is referred to as glutathione *S*-transferase AA [10]. This latter transferase also is present in high concentration in rat testis [11]. The YaYa homodimer was isolated by Carne *et al.* [6] and Hayes *et al.* [4], who refer to it as ligandin or lithocholic acid binding protein. In addition to the transferases that contain Ya and/or Yc subunits, there are additional enzyme forms that are composed of subunits that are intermediate in molecular weight between Ya and Yc. These former subunits are referred to as Yb and are found in glutathione *S*-transferases A and C [11].

In the current study we have developed a purification scheme that allows for the rapid isolation of all five forms of rat liver glutathione *S*-transferase discussed above. Using these pure proteins, we have sought to determine how subunit structure affects both catalytic and binding functions, whether the lower molecular weight subunits Ya and Yb arise from Yc, and whether *in vitro* hybridization studies can explain the pattern of isozymes observed *in vivo*. In addition, we have performed hybridization studies with transferases AA (YcYc) isolated from rat testes and YaYa isolated from rat liver to examine the question of organ specificity.

MATERIALS AND METHODS

Materials. 1-Chloro-2,4-dinitrobenzene and sodium lauryl sulfate were products of BDH Chemicals, Poole, England. The Sigma Chemical Co., St. Louis, MO, was also the source for the latter compound as well as for sulfobromophthalein, reduced glutathione (GSH), NADPH, glutathione reductase (EC 1.6.4.2), ethacrynic acid, bilirubin, *trans*-4-phenyl-3-buten-2-one, TEAE cellulose, acrylamide, *N*,*N*′-methylene-bis-acrylamide, carbonic anhydrase, chymotrypsinogen A, soybean trypsin inhib-

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itor, and epoxy-activated Sepharose. Sodium myristyl sulfate was obtained from the Eastman Kodak Co., Rochester, NY. Cumene hydroperoxide was obtained from ICN Pharmaceuticals Inc., Plainview, NY, and indocyanine green from Pfaltz & Bauer Inc., Stamford, CT. 1,2-Dichloro-4-nitrobenzene and *p*-nitrobenzyl chloride were obtained from the Aldrich Chemical Co., Milwaukee, WI, and were recrystallized from ethanol–water before use. DE-52 and CM-52 cellulose was purchased from Whatman, Inc., Clifton, NJ.

Buffers. Buffer A: Tris–HCl, 10 mM, pH 9.3, 4°; Buffer B: Tris—HCl, 40 mM, pH 8.0, 4°; Buffer C: potassium phosphate, 10 mM, pH 6.7; Buffer D: potassium phosphate, 10 mM, pH 6.7, 30% (w/v) glycerol, 1 mM EDTA.

Spectrophotometric enzyme assay. Glutathione *S*-transferase and glutathione peroxidase activities were determined as described previously [12, 13]. All activities were corrected for non-enzyme rates and are expressed as μmoles/min. Protein was determined by the method of Lowry *et al.* [14].

Preparation of enzymes. The glutathione *S*-transferases were prepared from livers of eight to twelve fed, male Sprague–Dawley rats, 250–300 g (Simonsen Laboratories, Gilroy, CA). The 100,000 *g* supernatant fraction was applied to and eluted from a DE-52 column as described previously [12]. The eluant was concentrated with the aid of a hollow fiber system and dialyzed against buffer A overnight. The sample was then applied to a TEAE cellulose column (2 × 23 cm) that had been equilibrated with buffer A. The column was washed with buffer A until no further transferase activity was present in the eluent (Fraction 1). The TEAE column then was washed with buffer B until no further enzymatic activity was present in the eluent (Fraction 2). Both fractions were concentrated over a stirred Amicon cell. The two fractions were then applied separately to a GSH affinity column (2.0 × 4.7 cm) equilibrated with 25 mM sodium phosphate, pH 9.4 [15]. The column was washed with 50 ml of 25 mM sodium phosphate, pH 9.4, that contained 150 mM NaCl. The transferases were eluted with 200 ml of 25 mM sodium phosphate, pH 9.4, that contained 150 mM NaCl and 100 mM GSH. The two TEAE fractions were concentrated over an Amicon cell and dialyzed for 36 hr against buffer C. They were applied separately to and eluted from a CM-cellulose column (2.5 × 40 cm) as described previously [12]. Three peaks of transferase activity were identified in TEAE Fraction 1 and two peaks in TEAE Fraction 2. The fractions containing these five transferases were pooled separately, concentrated, dialyzed against buffer D, and stored at −80°. The purification of transferase YcYc from testis was essentially as described above except that the TEAE cellulose column was not necessary as the YcYc enzyme from the testis was free of contamination by other transferases following elution from the CM-cellulose column.

Sodium dodecyl sulfate gel electrophoresis and electrofocusing. The glutathione *S*-transferases were subjected to discontinuous sodium dodecyl sulfate gel electrophoresis as described by Maizel [16]. Protein standards were carbonic anhydrase, mol. wt = 29,000; chymotrypsinogen A, mol. wt = 25,700; and soybean trypsin inhibitor, mol. wt = 21,000. Electrofocusing was performed in polyacrylamide gels as described previously [17], with the exception that, for certain experiments, the ampholine solution consisted of a 1/1 (v/v) mixture of 40% (w/v), pH 3.5–10 range, and 20% (w/v), pH 9–11 range, carrier ampholytes.

Binding studies. Binding studies were performed at 25° using a Perkin–Elmer MPF-44B fluorescence spectrophotometer with a temperature-controlled cell compartment. The cuvettes contained 10% (v/v) glycerol; 0.3 mM EDTA; 1 mM GSH; 0.1 M potassium phosphate, pH 7.5; and 0.5 to 10 μM protein in a final volume of 3 ml. Bilirubin was dissolved in 10 mM NaOH, and all solutions were protected from light. The ligands were added sequentially (maximum of 300 μl total) to the cuvette, and the solution was stirred following each addition. The decrease in fluorescence following addition of ligand was monitored at 320 nm with excitation at 285 nm [18]. Corrections were made for dilution and absorption of incident and fluorescent light [19]. The K_D values were determined from a double-reciprocal plots of the change in fluorescence versus the concentration of free ligand. The final concentration of ligand did not exceed 10 μM. The stoichiometry of binding of bilirubin and indocyanine green by the YaYa transferase was evaluated as described previously [20]. The number of binding sites was determined by Scatchard analysis [21].

Hybridization of glutathione S-transferases. These studies followed the approach described by Habig *et al.* [10]. Approximately equal molar amounts of the two transferases were incubated at 25° in 50 mM potassium phosphate, pH 7.5, containing 20 mM 2-mercaptoethanol, and 6 M guanidine–HCl. After 1 hr, this mixture was diluted 9-fold with 5 mM potassium phosphate, pH 6.7, 0.1 mM EDTA, and 25% (v/v) glycerol and incubated for another hour at 25°. The mixture was then dialyzed at 4° versus this buffer for 36 hr (buffer was changed five times). For the final dialysis, phosphate concentration was reduced to 1 mM. Following the final dialysis, the enzyme activity was 70–80% of the starting activity. There was no visible precipitate. The enzyme solution was applied to a column of CM-cellulose (0.7 × 10 cm) equilibrated with 1 mM potassium phosphate, pH 6.7, 0.1 mM EDTA, and 25% (v/v) glycerol. The column was washed with two column volumes of a buffer containing 5 mM phosphate, 0.1 mM EDTA, and 25% (v/v) glycerol, and then developed with this buffer containing a linear gradient of 0–75 mM KCl in a total gradient volume of 100–150 ml. Fractions of 2 ml were collected and analyzed for enzyme activity.

Test for conversion of Yc subunit to Ya subunit. Glutathione *S*-transferase AA (YcYc) purified from rat liver or testis was used for these studies. The purified transferase (0.2 mg/ml) was incubated with or without 0.013 to 0.025 mg/ml of rat liver 100,000 *g* supernatant fraction in 10 mM potassium phosphate, pH 6.7, 28% (v/v) glycerol and 0.003% sodium azide for up to 1 week at both 0° and at room temperature. The solutions were subjected to sodium dodecyl sulfate gel electrophoresis.

Tryptophan analysis. The purified transferases were analyzed for their tryptophan content as described previously [22].

RESULTS

Preparation and identification of purified transferases. Five enzymes forms were identified and purified, three of which were in Fraction 1 from the TEAE cellulose column and two in Fraction 2. When subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the three enzymes in Fraction 1 were shown to be two homodimers, YaYa and YcYc, and transferase B (YaYc). Fraction 2 contained two homodimers of the Yb subunit (Fig. 1). The transferase isolated from rat testis was, as expected, a YcYc homodimer. The pI values of the transferases composed of the Ya and/or Yc subunits (including testis) were essentially identical and in the range of pI = 9.5 to 9.7. The two transferases composed of Yb subunits had pI values of 8.8 and 8.0 respectively. The subunit structures and pI values of the latter two transferases were identical to glutathione S-transferases A and C respectively [11, 12].

When sodium dodecyl sulfate gel electrophoresis was performed using lauryl sulfate purchased from the Sigma Chemical Co., the Ya subunit was resolved into two bands (not shown). If the source of lauryl sulfate was BDH Chemicals, then Ya was a single band (Fig. 1). Lauryl sulfate from the Sigma Chemical Co. contains 26% myristyl sulfate. When sodium dodecyl sulfate gel electrophoresis was performed with a 3/1 (w/w) mixture of lauryl sulfate and myristyl sulfate, the Ya subunit of both glutathione S-transferase B and YaYa was subfractionated into two subunits that differ slightly in molecular weight (Fig. 2). At ratios of 19/1 and 9/1 (lauryl/myristyl), the Ya subunit appeared homogeneous. The Yb and Yc subunits were homogenous irrespective of the amount of myristyl sulfate present (Figs. 1 and 2).

Substrate specificities. Table 1 summarizes the substrate specificities of various transferases. Those isozymes containing the Ya and/or Yc subunit had similar substrate specificities in that they had high enzymatic activity with 1-chloro-2,4-dinitrobenzene as acceptor but relatively low activity with 1,2-dichloro-4-nitrobenzene, ethacrynic acid, *p*-nitrobenzyl chloride, sulfobromophthalein, or *trans*-4-phenyl-3-buten-2-one as acceptor. The latter five compounds, on the other hand, were good substrates for transferases containing the Yb subunits. These latter transferases had little activity with cumene hydroperoxide, i.e. function as glutathione S-peroxidases, but transferases YaYa, B (YaYc), and AA (YcYc, liver or testis) had substantial peroxidase activity. The activity of YaYa was about half of that present in transferase B (YaYc) and one quarter of that present in AA liver (YcYc) (Table 1), indicating that the subunit composition plays a role in the expression of the peroxidase activity.

Binding studies. Each transferase tested bound the ligands with K_D values of less than 5 μM (Table 2).

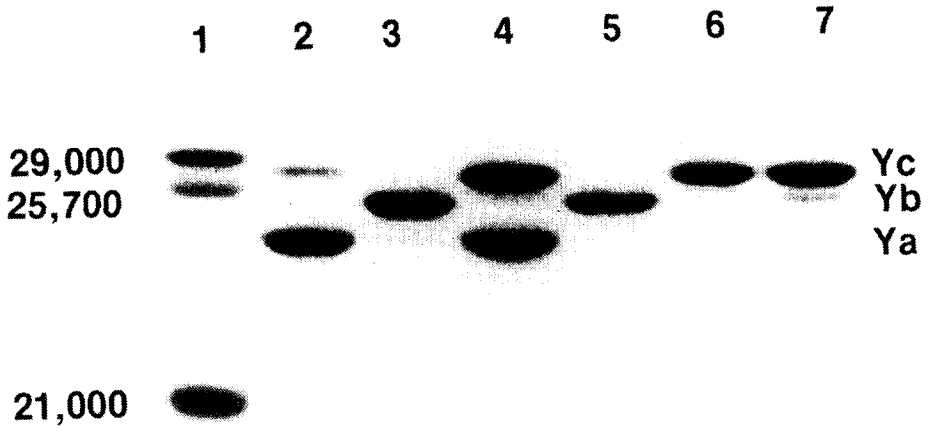

Fig. 1. Sodium dodecyl sulfate gel electrophoresis of the purified rat liver transferases. The lauryl sulfate was free of contamination by other fatty acyl sulfates. Channels 1–7 contained, respectively: standards (chymotrypsinogen A, carbonic anhydrase, soybean trypsin inhibitor) and transferases YaYa, C (YbYb), B (YaYc), A (YbYb), AA (YcYc-liver), and AA (YcYc-testis).

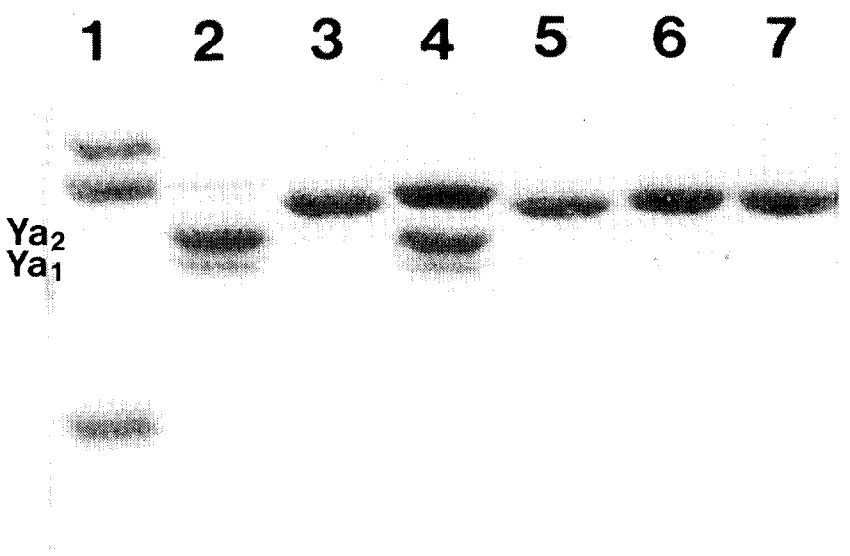

Fig. 2. Sodium dodecyl sulfate gel electrophoresis of the purified rat liver glutathione *S*-transferases using a 3:1 mixture of lauryl and myristyl sulfate. The contents of the channels were identical to Fig. 1.

Table 1. Substrate specificities of glutathione *S*-transferases

	Glutathione *S*-transferase activity (μmoles · min^{-1} · mg^{-1})				
	Form of transferase				
Substrate*	AA (YcYc)	Testis (YcYc)	B (YaYc)	YaYa	C (YbYb)
1-Chloro-2,4-dinitrobenzene	23.4	14.9	24.4	22.7	31.1
Ethacrynic acid	0.55	0.91	0.51	0.4	0.19
1,2-Dichloro-4-nitrobenzene	0.041	0.073	0.078	0.096	3.28
p-Nitrobenzyl chloride	0.56	0.27	0.54	0.72	8.00
Sulfobromophthalein	0.005	0.017	0.021	0.013	0.45
Trans-4-phenyl-3-buten-2-one	0.01	0.01	0.005	0.016	0.89
Cumene hydroperoxide	8.0	3.2	4.1	2.2	0.2

* The conditions of the assays were identical to those described previously [11, 12].

The values obtained for glutathione *S*-transferase B (YaYc) and YaYa were similar, whereas the K_D values obtained for glutathione *S*-transferase AA (YcYc) were 3.9- to 12-fold greater (Table 2).

The stoichiometry of binding of bilirubin and indocyanine green to glutathione *S*-transferase YaYa is shown in Fig. 3. Glutathione *S*-transferase YaYa bound one mole of bilirubin or indocyanine green per mole enzyme. K_D values from the Scatchard plots were 0.51 and 1.2 μM for bilirubin and indocyanine green respectively. These values are similar to those obtained using quenching of fluorescence at a single concentration of protein (Table 2).

Hybridization studies. Mixing glutathione *S*-transferases AA (YcYc, liver) and YaYa in the absence of guanidine–HCl followed by chromatography on CM-cellulose failed to show the formation of a heterodimer. Mixing the two homodimers in 6 M guanidine–HCl, as described in Materials and Methods, and then applying the mixture to a CM-cellulose column yielded three enzymes forms in a ratio of approximately 1:2:1 (Fig. 4). The large middle peak

Table 2. Binding of ligands by glutathione *S*-transferases

	K_D* (μM)	
	Ligand	
Form of transferase	Bilirubin	Indocyanine green
AA (YcYc)	4.1	3.69
B (YaYc)	0.68	0.94
YaYa	0.34	0.55

* The K_D values were determined from double-reciprocal plots of the change in fluorescence versus the concentration of free ligand.

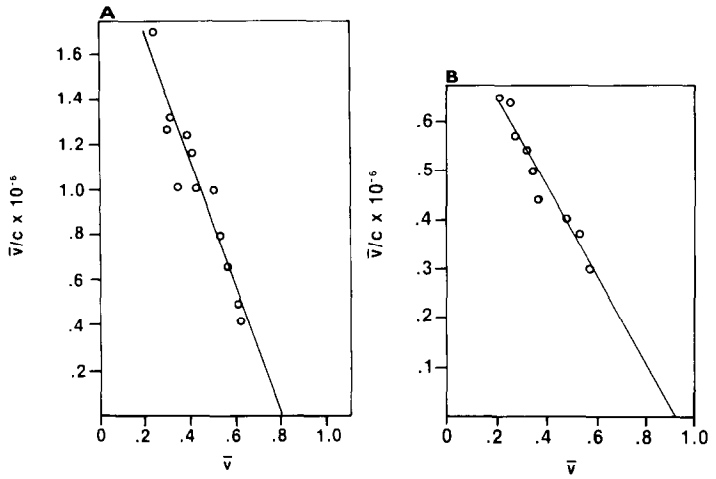

Fig. 3. Stoichiometry of binding of bilirubin (A) and indocyanine green (B) by transferase YaYa. The number of binding sites was determined by the method of Halfman and Nishida [20].

of enzyme activity was a YaYc heterodimer as determined by gel electrophoresis, whereas the two other peaks of activity were homodimers of Ya or Yc (Fig. 4). In addition to the reconstitution of transferase activity after treatment with guanidine–HCl, glutathione peroxidase activity also was present. The newly formed YaYc hybrid had the level of peroxidase activity expected for this heterodimer (Table 1).

Glutathione S-transferase B (YaYc), when treated with guanidine–HCl and then chromatographed on CM-cellulose, yielded three peaks of enzyme activity that were essentially identical to those shown in Fig. 4. The YaYa hybrid relative to the original YaYc heterodimer had reduced peroxidase activity as expected, based on data in Table 1. In contrast, the peroxidase activity in the YcYc hybrid was greater. The ratios of transferase activity, assayed with 1-chloro-2,4-dinitrobenzene, and peroxidase activity in the native proteins AA (YcYc), B (YaYc) and YaYa were 2.9:1, 6:1 and 10.3:1 respectively (Table 1). In the hybrids (Fig. 4) these same ratios were 2.7:1, 4.6:1 and 12:1 respectively. Thus, the functional characteristics of the hybrids were similar to the native proteins.

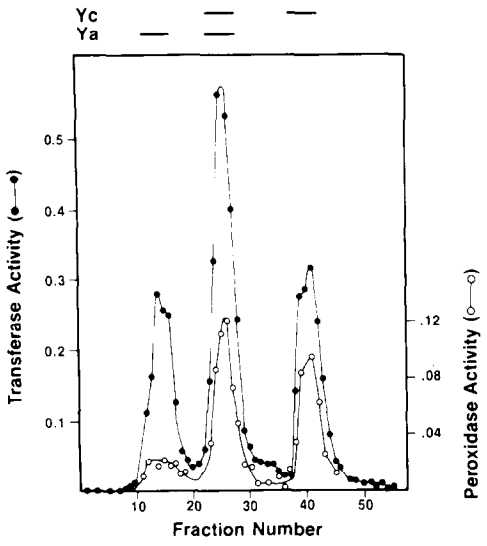

Fig. 4. CM-cellulose chromatography of liver transferases AA (YcYc) and YaYa that were treated with 6 M guanidine–HCl. Glutathione S-transferase activity (●) was assayed with 1-chloro-2,4-dinitrobenzene and peroxidase activity (○) with cumene hydroperoxide. Enzyme activity is in μmoles per min per ml. The subunit composition of each transferase was determined as described in Materials and Methods.

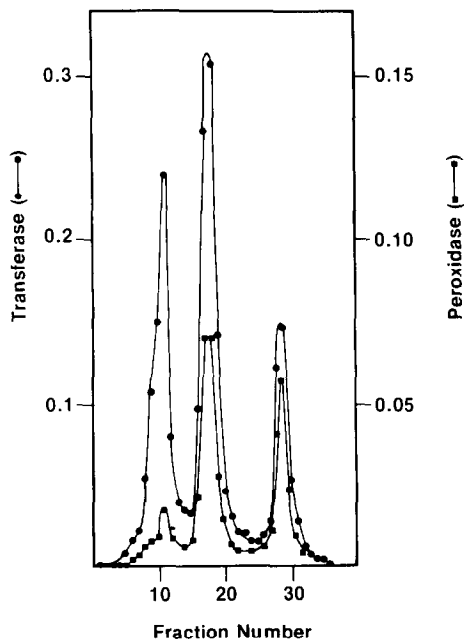

Fig. 5. CM-cellulose chromatography of liver transferase YaYa and testis transferase AA (YcYc) that were treated with guanidine–HCl and renatured. Glutathione S-transferase activity (●) was assayed with 1-chloro-2,4-dinitrobenzene and peroxidase activity (■) with cumene hydroperoxide. The middle peak of transferase activity was a YaYc heterodimer.

Table 3. Tryptophan content of glutathione *S*-transferases

Form of transferase	Residues per mole*
AA (YcYc)	4
B (YaYc)	3
YaYa	3
A (YbYb)	8
C (YbYb)	7

* The subunit molecular weights were assumed to be: Ya = 22,000; Yb = 24,000; and Yc = 25,000.

Glutathione *S*-transferase YaYa from liver and AA (YcYc) from testis also were mixed in 6 M guanidine–HCl. Following chromatography on CM-cellulose, three peaks of enzyme activity were obtained (Fig. 5), and the middle peak was a YaYc heterodimer. This demonstrates the similarity of the YcYc transferase from both liver and testis.

Hybrids between glutathione *S*-transferase YaYa and A (YbYb) were not formed by guanidine-HCl treatment, in contrast to Ya and Yc subunits, suggesting that they lack the necessary determinants to form a heterodimer.

Interconversion of subunits. Investigators have suggested that the Ya and Yb subunits are proteolytic products of Yc [11]. Glutathione *S*-transferase YaYa also is believed to form during the storage of YaYc [9]. We tested this idea by incubating purified glutathione *S*-transferase AA (YcYc) from rat liver and testis in the presence and absence of 100,000 *g* supernatant fraction from rat liver, as described in Materials and Methods. No conversion of Yc to Ya or to Yb was observed during the duration of the experiment (up to 1 week at room temperature). Also, during storage of the purified transferases AA (YcYc) and B (YaYc) (several months), there was no redistribution of subunits.

Tryptophan content of glutathione S-transferases. The amino acid compositions of the various transferases are very similar [1]. One striking difference, however, is the tryptophan content. Transferases AA (YcYc), A (YbYb), B (YaYc) and C (YbYb) are reported to have 2, 6, 9, and 6 residues of tryptophan/mole of transferase respectively [1]. No value has yet been reported for the tryptophan content of transferase YaYa. Thus, if each subunit of transferase AA (YcYc) contained one residue of tryptoplan and the Yc subunit of this isozyme were identical to the Yc subunit of transferase B (YaYc), then it would be predicted that the Ya subunit would contain 8 residues of tryptophan and consequently transferase YaYa should have 16 residues/mole. Moreover, the Ya subunit could not arise from the Yc subunit based on this data. We found, on the other hand, that the tryptophan contents of transferases AA (YcYc), B (YaYc) and YaYa were similar (Table 3). The corresponding values for transferases A (YbYb) and C (YbYb) were higher and in close agreement with those previously reported [1].

DISCUSSION

The glutathione *S*-transferases have been purified from rat liver by a variety of techniques. The method used most frequently is that of Habig *et al.* [12]. Using this method, six forms of glutathione *S*-transferase were identified previously; however, the YaYa transferase was not [10, 12]. We observed that the YaYa protein was present in the "C" peak of the CM-cellulose column when isolating the transferases as described by Habig *et al.* [12]. The method of Arias *et al.* [23] has been used by several investigators to purify ligandin. This preparation, however, contains a mixture of both the YaYa and YaYc transferases unless it is applied to a CM-cellulose column [4]. Strange and colleagues [24] have described a method for purifying the YaYa and YaYc transferases; however, the other forms of glutathione *S*-transferase are not obtained with the methods of either Arias [23] or Strange [24]. Using the method described in the current investigation, five forms of glutathione *S*-transferase were purified from the rat liver with essentially no cross-contaminaiton between enzyme forms. Also, additional enzymes, probably the D and E transferases, were present in the second fraction eluted from the TEAE cellulose column. Thus, all forms of the glutathione *S*-transferases present in rat liver could be purified with relative ease using the method described in this paper.

The origin of the Ya subunit is uncertain. Conversion of YaYc to YaYa has been reported to occur duing storage of the YaYc protein in some [9, 25] but not other [5, 8] studies. One group of investigators has suggested that both the Ya and Yb subunits arise from Yc due to proteolysis that occurs either *in vivo* or during isolation of the proteins [11]. Proteolysis during isolation is unlikely to account for the different isozymes obtained, as the addition of proteinase inhibitors has failed to reduce the number of isozymes observed [8, 9]. In agreement with these latter studies, we found no conversion of the purified YcYc protein from either liver or testis to other forms over the course of 1 week in the presence or absence of hepatic cytosol, suggesting a lack of specific proteases from this source. Also, the tryptophan content of Yb relative to Yc was inconsistent with the suggestion that Yb arises from Yc (Table 3). This argument cannot be used to eliminate the possibility that Ya arises from Yc. On the other hand, in cell-free translation systems, both the Ya and Yc subunits are synthesized [25–27]; Ya synthesis is increased relative to Yc synthesis after phenobarbital treatment [26, 27], suggesting that the two subunits are synthesized from two different mRNAs.

In the current investigation, the Ya subunit could be resolved into two components when the ratio of lauryl sulfate to myristyl sulfate was 3:1 (Fig. 4) or when certain sources of sodium dodecyl sulfate were used. Such microheterogeneity has been observed for some other proteins but has not been reported previously with the glutathione *S*-transferases. For example, the subunit composition of tubulin obtained by sodium dodecyl sulfate gel electrophoresis depends upon the composition of the detergent used [28]. The resolution associated with different detergents is believed to reflect different affinities of the subunits for the detergents. The microheterogeneity of the Ya subunit suggests that transferase B is a mixture of Ya_1Yc and Ya_2Yc, whereas transferase YaYa may be, in part, a Ya_1Ya_2 heterodimer.

Table 4. Classification of glutathione S-transferases of rat liver

Characteristic	Class A	Class B
Name*	A and C	ligandin, B, AA
Subunit structure	YbYb	YaYa, YaYc, YcYc
pI value	8.8 and 8.0	9.5 to 9.7
Binds TEAE, pH 9.4, 4°	Yes	No
Activity with:		
1,2-Dichloro-4-nitrobenzene	High	Low
Organic hydroperoxides	Low	High
Affinity for non-substrate ligands	High†	High

* Name of individual transferases used in other publications [2, 3, 12].
† Based on the work of Ketley *et al.* [18].

Previous investigators have attempted to form hybrids of glutathione S-transferase by mixing different transferases together. Some investigators failed to find hybrid formation [9, 10], whereas in other studies hybridization was observed [8, 29]. In the current study, we found that glutathione S-transferase YaYa, when mixed with YcYc from either liver or testes, yielded glutathione S-transferase YcYc, YaYc, and YaYa in the expected ratio of 1:2:1 (Fig. 4). In previous studies [8, 29], this ratio was not observed, perhaps because dialysis to remove the denaturant was undertaken at higher protein concentration. In addition, in the current studies, the hybrids have been shown to be functionally similar to the native proteins, i.e. they contain both transferase and peroxidase activity. These data are consistent with the idea that glutathione S-transferase B (YaYc) is an *in vivo* hybrid of the subunits of transferases AA (YcYc) and YaYa. In contrast, when transferase YaYa and A (YbYb) were mixed, no rearrangement occurred. The absence of YaYb and YcYb hybrids *in vivo* is consistent with observations that they do not form *in vitro*.

In the current study, the K_D values for bilirubin (Table 2) and the YaYc and YaYa transferases were quite similar to previous reports [9, 19, 30, 31]. On the other hand, the K_D values for indocyanine green and transferase YaYc were somewhat lower than the values reported by Ketley *et al.* [18]. The K_D values obtained with the YcYc transferase (Table 2) were quite different from those reported previously. Ketley and colleagues [18] reported K_D values of 100 μM for bilirubin and indocyanine green using the YcYc transferase isolated from rat liver. The reasons for these differences are unclear; however, based on our investigations, all three transferases that contain Ya and/or Yc subunits had a relatively high affinity for these non-substrate ligands (Table 2).

Transferase B has been reported to contain a single high-affinity binding site for bilirubin [7, 18]. It has been suggested that the high-affinity binding site resides on the Ya subunit and that the YaYa protein has two binding sites [9, 32]. Reports by other investigators, however, have identified only a single binding site for bilirubin on the YaYa protein [31]. In the current study, the YaYa transferase contained only a single binding site for either bilirubin or indocyanine green (Fig. 3). Thus, it is unlikely that the Ya subunit alone determines the nature of the binding of non-substrate ligands by transferase B (YaYc).

The characteristics of the liver transferases purified in this study allow then to be separated into two classes based on their subunit structures, isoelectric points, and substrate specificities (Table 4). The "A" class is representative of transferases A (YbYb) and C (YbYb) and can be distinguished from the "B" class (YaYa, YaYc, YcYc) by the former's binding to TEAE cellulose (pH 9.4, 4°), pI values and substrate specificities (high activity with 1,2-dichloro-4-nitrobenzene and low peroxidase activity). Additional transferases present within rat liver, i.e. D and E, do not fall within the characteristics of the above two classes.

Since the original submission of this work, other investigators have published evidence which corroborates some of our findings. Specifically, Mannervik and Jensson [33] have isolated similar forms of the glutathione S-transferases using a somewhat different purification scheme. The substrate specificities of the latter isozymes are in agreement with the results obtained in this paper.

Acknowledgements—This work was supported in part by the Veterans Administration and by grants from the National Science Foundation (PCM-7817706 and PCM-8108283). The excellent technical assistance of Cherie Holcomb and Jerryl Neher is gratefully acknowledged.

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