Purification and characterization of glutathione S-transferases P, S and N

Isolation from rat liver of Yb₁Yn protein, the existence of which was predicted by subunit hybridization *in vitro*

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The glutathione S-transferases are dimeric proteins and comprise subunits of M_r 25 500 (Ya), 26 500 (Yn), 27 000 (Yb₁ and Yb₂) and 28 500 (Yc). Enzymes containing Ya and/or Yc subunits have been isolated as have forms containing binary combinations of Yn, Yb₁ and Yb₂ subunits. To date only one enzyme, transferase S, has been described that is a YbYn heterodimer [Hayes & Chalmers (1983) *Biochem. J.* **215**, 581–588]; the identity of the Yb monomer found in transferase S has not been reported previously. The identification and isolation of a YnYn dimer (transferase N) from rat testis is now described. This has enabled structural and functional comparisons to be made between Yb₁, Yb₂ and Yn monomers. Reversible dissociation experiments between the YnYn and Yb₁Yb₁ homodimers and between the YnYn and Yb₂Yb₂ homodimers demonstrated that Yn monomers can hybridize with both Yb₁ and Yb₂ monomers. Reversible dissociation of transferases N and C (Yb₁Yb₂) showed that both Yb₁ and Yb₂ monomers can hybridize with Yn monomers under competitive conditions. The hybridization data suggest that transferase S represents the Yb₂Yn subunit combination. A knowledge of the elution position from chromatofocusing columns of the Yb₁Yn hybrid that was formed *in vitro* enabled a purification scheme to be devised for an enzyme from rat liver (transferase P) believed to consist of Yb₁Yn subunits. A comparison of the chromatographic behaviour of the YnYn, Yb₁Yb₁ and Yb₂Yb₂ dimers on chromatofocusing and hydroxyapatite columns with the behaviour of transferases P and S on the same matrices suggests these two enzymes may be identified as the Yb₁Yn and Yb₂Yn dimers respectively. The catalytic activities and the inhibitory effects of non-substrate ligands on transferases P and S are significantly different and again suggest they comprise Yb₁ and Yn subunits and Yb₂ and Yn subunits respectively; transferase P exhibits a 6-fold higher specific activity for 1,2-dichloro-4-nitrobenzene than does transferase S, whereas, conversely, transferase S possesses a 9-fold higher specific activity for *trans*-4-phenylbut-3-en-2-one than does transferase P. The quaternary structure of transferases P and S was verified by using peptide mapping and 'Western blotting' techniques.

The glutathione S-transferases are a 'multi-gene' family of detoxication proteins that provide protection against lipophilic electrophiles (Jakoby, 1978).

The transferases each comprise two of a limited

Abbreviations used: GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one; SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography.

number of subunits each of which function autonomously (Jakobson *et al.*, 1977). The nomenclature used to describe these monomers is based on the work of Bass *et al.* (1977), who reported that the 'Y fraction', which is obtained by gel-filtration chromatography and contains partially purified transferases, is enriched with three polypeptides, designated Ya (M_r 25 500), Yb (M_r 27 000) and Yc (M_r 28 500). In the present paper the molecular mass of the Ya, Yn, Yb and Yc monomers has been

re-calculated on the basis of the data presented by Pickett *et al.* (1984) and Lai *et al.* (1984), which demonstrate that a full-length clone of the Ya subunit codes for a polypeptide(s) of M_r 25500. It has subsequently been shown that there are at least two distinct transferase monomers of apparent molecular mass 27000Da (i.e. Yb₁ and Yb₂), and YaYa, YaYc, YcYc, Yb₁Yb₁, Yb₁Yb₂ and Yb₂Yb₂ subunit combinations exist in rat liver (Bhargava *et al.*, 1978; Hayes, 1980, 1983; Beale *et al.*, 1982, 1983; Mannervik & Jensson, 1982; Frey *et al.*, 1983).

Mannervik & Jensson (1982) showed that individual monomers exhibit characteristic activities. For example, the Yb₁ and Yb₂ subunits are catalytically distinguishable, as the Yb₁ subunit displays a high activity for DCNB but is inactive towards tPBO, whereas, conversely, the Yb₂ subunit has high activity for tPBO but is unable to conjugate DCNB with GSH.

Recently it has been shown that an additional transferase subunit exists in the rat. This monomer (Yn) migrates between the Ya and Yb subunits during SDS/polyacrylamide-gel electrophoresis and has a molecular mass of 26500Da (Hayes & Chalmers, 1983). The relationship between the Yn and Yb monomers is of particular interest, since the Yn monomer was originally recognized as one of the subunits of transferase S; this enzyme was shown to be a YbYn dimer, but the type of Yb monomer represented was not established.

Transferase S was originally isolated from rat liver, and, although this organ contains appreciable amounts of the Yb₁ and Yb₂ homodimers, little YnYn protein can be recovered from hepatic extracts. A purification scheme for YnYn protein (transferase N) from rat testis is now described. The availability of homogeneous preparations of YnYn has enabled experiments to be undertaken to determine whether both Yb₁Yn and Yb₂Yn dimers exist; to date only one Yn-containing heterodimer has been purified. Hybridization experiments between YnYn protein and Yb₁Yb₂ protein have been performed to allow the ability of the Yb₁ and Yb₂ subunits to compete simultaneously for binding to the Yn subunit to be assessed. The purification from rat liver of a second YbYn-containing enzyme, transferase P, which has not been previously isolated, is described. Transferases P and S were characterized and evidence is presented that they represent the hepatic Yb₁Yn and Yb₂Yn heterodimers respectively.

Materials and methods

Chemicals

These are of analytical grade and were readily available commercially. The h.p.l.c.-grade sol-

vents and sequencer-grade trifluoroacetic acid were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland.

Buffers

All buffers, with the exception of those used in the enzyme assays, were prepared at 4°C.

Analytical methods

The electrophoretic methods and enzyme assays have been described previously (Hayes, 1980, 1983; Hayes & Clarkson, 1982). The h.p.l.c. system employed in the present study was from Waters Associates (Instruments), Northwich, Cheshire, U.K., and comprised two model 510 pumps, a model 680 automated gradient controller, a model 481 Lambda-Max absorbance detector and a model U6K universal injector. The solvent systems used during the h.p.l.c. separation of peptides have been described by Hermondson & Mahoney (1983).

Purification of glutathione S-transferases

Transferases A, B and C were purified from rat liver by the method of Habig *et al.* (1974). Transferases D and S were prepared as described previously (Hayes, 1983; Hayes & Chalmers, 1983).

Enzyme assays

Glutathione S-transferase activity with CDNB or DCNB was measured at 37°C by using a centrifugal analyser (Hayes & Clarkson, 1982). Assays performed with tPBO were carried out at 37°C manually.

Experimental and results

During the present study two main experimental approaches were used to determine the relationship between the Yb and Yn monomers. Firstly, the possibility that both Yb₁Yn and Yb₂Yn heterodimers exist in rat liver was explored by using hybridization techniques. Secondly, the identity of the Yb subunit that two YbYn proteins contained was established by comparing their catalytic activities with those of the Yb₁Yb₁, Yb₂Yb₂ and YnYn dimers and by using peptide-mapping methods. To enable either of these approaches to be viable a source of YnYn protein was sought.

Identification of Yn polypeptides in extrahepatic tissue and the purification of YnYn protein

Rat liver contains little YnYn protein, and therefore a survey of extra-hepatic organs was undertaken to identify a suitable tissue as a possible source of the Yn dimer. The 100000g supernatants from kidney, spleen and testis were

each subjected to *S*-hexylglutathione affinity chromatography (Mannervik & Guthenberg, 1981). The transferases that were purified by this step were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). This demonstrated that rat testis contained appreciably more Yn polypeptide(s) than did kidney or spleen and therefore represented the best source of YnYn protein among the tissues tested.

The YnYn-containing enzyme (transferase N) was purified from rat testis by using a combination of *S*-hexylglutathione affinity chromatography, chromatofocusing and hydroxyapatite chromatography. The chromatographic properties of this enzyme are summarized in Table 1.

Hybridization of transferase N with transferases A and D

Experiments were performed to determine whether the Yn monomer could form hybrids *in vitro* with both the Yb₁ and Yb₂ subunits. Equimolar mixtures of YnYn protein and transferase A (Yb₁Yb₁) or of YnYn protein and transferase D (Yb₂Yb₂) were denatured in 6M-guanidinium chloride and re-associated as described previously (Hayes, 1983). Chromatographic analysis of the products obtained after the reversible denaturation of these subunits showed that both Yb₁ and Yb₂ monomers can hybridize with Yn monomers (Fig. 2).

Dissociation and self-assembly of transferase S

Fig. 3 shows the profile obtained from hepatic transferase S after reversible denaturation with guanidinium chloride. The three peaks of activity that were resolved by chromatofocusing were eluted at pH 8.13, 7.92 and 7.70. The first of these peaks catalysed the conjugation of CDNB, but not of DCNB, with GSH. The enzymes in the peaks eluted at pH 7.92 and 7.70 were active with both CDNB and DCNB as substrates. Analysis of the enzyme-containing fractions by SDS/polyacrylamide-gel electrophoresis demonstrated that the

Fig. 1. Identification of Yn polypeptides in rat testis cytosol. Dialysed 100 000g supernatants from various extra-hepatic tissues were applied to *S*-hexylglutathione-agarose columns, and the material that was specifically eluted was analysed by SDS/polyacrylamide-gel electrophoresis (12.5% polyacrylamide resolving gel). Portions of the purified glutathione *S*-transferase fractions were applied to the gel as follows: lane 1, 25 µg of kidney transferases; lane 2, 10 µg of spleen transferases; lane 3, 20 µg of testicular transferases; lane 4, 15 µg of hepatic transferase S (YbYn). The positions of the Ya, Yn, Yb and Yc subunits are indicated. The polypeptide in kidney that migrates faster than the Ya subunit is labelled 'Yf'.

Table 1. Electrophoretic and chromatographic properties of Yb- and Yn-containing glutathione transferases. For full experimental details see the text. Chromatofocusing was performed with 1.6 cm × 38.0 cm columns of PBE 94. Hydroxyapatite chromatography was carried out in 1.6 cm × 19.0 cm columns and the 10–250 mM-sodium phosphate gradient was formed at pH 6.7 by using a 200 ml constant-volume mixing reservoir.

Transferase	pH eluted from chromatofocusing column	[Na ⁺] eluted from hydroxyapatite column (mM)	10 ⁻³ × Subunit M _r	Subunit composition
A	8.8–8.6	295	27.0/27.0	Yb ₁ Yb ₁
C	8.4–8.25	255	27.0/27.0	Yb ₁ Yb ₂
D	8.1	200	27.0/27.0	Yb ₂ Yb ₂
P	8.3	273	27.0/26.5	Yb ₁ Yn
S	7.9	198	27.0/26.5	Yb ₂ Yn
N	6.6	185	26.5/26.5	YnYn

Fig. 2. *Chromatographic properties of the Yb₁Yn and Yb₂Yn hybrids formed after reversible denaturation* Equimolar mixtures of (a) YnYn and Yb₁Yb₁ proteins or (b) YnYn and Yb₂Yb₂ proteins, 1 mg of each, were reversibly denatured with 6M-guanidinium chloride. The products obtained were analysed by chromatofocusing to allow the elution positions of the re-associated Yb₁Yn and Yb₂Yn proteins to be compared with the hybrid obtained after renaturation of guanidinium chloride-treated transferase S (see Fig. 3). The PBE 94 chromatofocusing columns (1.6cm × 38.0cm) were eluted at 18 ml/h, and 7.0 ml fractions were collected. The pH (●) of the eluate was measured and transferase activity was determined with CDNB (▲). The protein in the enzyme-containing fractions was concentrated by precipitation with 10% (w/v) trichloroacetic acid (0°C) and subjected to SDS/polyacrylamide-gel electrophoresis. The subunits that these fractions comprised are shown.

transferase eluted at pH 8.13 comprised Yb subunits, that the peak eluted at pH 7.92 contained YbYn polypeptides, and that the transferase eluted at pH 7.70 consisted of Yn subunits. As the Yb monomer possessed no DCNB-GSH-conjugating activity, it was considered highly likely that transferase S comprises Yb₂Yn subunits.

It is noteworthy that after guanidinium chloride treatment the YnYn protein (*M_r* 26500) derived from the hepatic transferase S and the YnYn protein (*M_r* 26500) derived from the testicular transferase N were both eluted at pH 7.7 from the chromatofocusing columns.

Evidence that Yn monomers can hybridize with Yb₁ and Yb₂ monomers under competitive conditions

Chromatographic analysis of the products ob-

tained after the reversible denaturation of transferase N (YnYn) with transferase C (Yb₁Yb₂) showed that five peaks of transferase activity could be resolved by chromatofocusing in a pH 8.5–6.5 gradient. SDS/polyacrylamide-gel electrophoresis demonstrated that two of these fractions contained YbYn protein whereas the remainder contained only Yb or Yn subunits. The peaks that contained both Yb and Yn polypeptides were eluted at positions similar to those shown in Fig. 2.

The re-association experiments demonstrated that, under competitive conditions, both the Yb₁ and Yb₂ subunits can form hybrids with the Yn subunit. This suggests that, in the absence of a post-translational mechanism responsible for directing hybrid formation, rat liver should contain both Yb₁Yn and Yb₂Yn heterodimers, since this organ contains a high concentration of the Yb₁ and Yb₂ polypeptides. Efforts were therefore made to isolate Yb₁Yn protein from rat liver.

Isolation of Yb₁Yn protein

The hybridization experiments demonstrated that the putative Yb₁Yn protein was eluted from a chromatofocusing column adjacent to transferase D (Yb₂Yb₂). This similarity in chromatographic behaviour was exploited in the initial purification step.

An enriched fraction of transferase D was prepared from rat liver 20000g supernatant by applying the hepatic extract (700 ml, 18 g of protein) to DEAE-cellulose (bed volume 2.2 litres) equilibrated with 10 mM-Tris/HCl buffer, pH 8.4. The transferases retained by this exchanger that were eluted by the NaCl gradient in the first peak of CDNB-GSH-conjugating activity ([Na⁺] 25–55 mM) were purified by S-hexylglutathione affinity chromatography (Mannervik & Guthenberg, 1981).

The material (210 mg) specifically eluted from S-hexylglutathione-Sepharose 6B was resolved by chromatofocusing into the individual isoenzymes (Fig. 4). Electrophoretic analysis of the enzyme-containing fractions demonstrated the existence of two pools of activity (at pH 8.3 and pH 7.9), which contained Yb and Yn subunits (Fig. 4). The YbYn-containing peak eluted at pH 7.9 was recovered in high concentration from the second CDNB-GSH-conjugating peak eluted from the DEAE-cellulose column ([Na⁺] 58–80 mM) and therefore represents transferase S (Hayes & Chalmers, 1983).

Purification of transferase P

The enzymes that were resolved by chromatofocusing were finally subjected to hydroxyapatite chromatography (Fig. 5), and the purity of each was assessed by SDS/polyacrylamide-gel electro-

Fig. 3. Analysis of the products obtained from hepatic glutathione transferase S after reversible denaturation. Portions of transferase S (about 2mg) were reversibly denatured with 6M-guanidinium chloride in 100mM-sodium phosphate buffer, pH6.5, containing 1mM-2-mercaptoethanol and 1mM-EDTA. After extensive dialysis against 25mM-Tris /acetate buffer pH8.8, the products were separated by chromatofocusing (pH range 8.4–6.0). The column (1.6cm × 38.0cm) was eluted at 18 ml/h, and 7.0 ml fractions were collected. Transferase activity was measured with CDNB (▲) and DCNB (○) as substrates. The pH (■) and absorbance at 280nm (●) were also measured.

Fig. 4. Isolation of glutathione transferase P by chromatofocusing. The material from DEAE-cellulose (equilibrated at pH8.4) that was eluted by NaCl (at [Na⁺] 25–55mM) was purified by S-hexylglutathione–Sephadex 6B chromatography as described in the text. A portion (210mg) of this purified transferase mixture was applied to a 1.6 cm × 37.0 cm column of chromatofocusing gel PBE 94 equilibrated with 25mM-Tris/acetate buffer, pH8.8. Fractions of volume 6.3 ml were collected, and transferase activity with CDNB (▲), DCNB (○) and tPBO (■) was determined. The pH (●) was measured in every fifth fraction. The subunit compositions of the enzyme-containing fractions were determined by SDS/polyacrylamide-gel electrophoresis and are indicated.

Fig. 5. Resolution of glutathione transferases by hydroxyapatite chromatography

The partially purified transferases, which had been subjected to DEAE-cellulose, *S*-hexylglutathione affinity and chromatofocusing chromatography, were finally dialysed against 10mM-sodium phosphate buffer, pH6.7, and applied to hydroxyapatite columns (1.6 cm $\times$ 19.0 cm) equilibrated with the same buffer. The columns, which were eluted at 21 ml/h, were developed with a 10–250mM-sodium phosphate gradient, which was formed by using a 200ml constant-volume mixing reservoir. Fractions of volume 4.2ml were collected and the Na⁺ concentration (■), the absorbance at 280nm (○) and transferase activity with CDNB (▲) were measured. Panels (a), (b) and (c) represent the hydroxyapatite elution profiles from the transferase P, C and S preparations (after chromatofocusing). The horizontal bars indicate the fractions that were combined and that represent the final purified products.

phoresis. Fig. 6 shows that transferase P contains two subunits with different apparent molecular masses; the larger monomer migrates with the Yb subunits, whereas the smaller monomer (Yn) migrates between the Ya and Yb subunits. It should be noted that transferases P and S (the two YbYn heterodimers), which are well resolved during the hydroxyapatite chromatography, show no evidence of interconversion during this last step, indicating that they are separate forms.

The chromatographic and electrophoretic properties of the major Yb- and Yn-containing enzymes, shown in Table 1, are consistent with the identification of transferases P and S as Yb₁Yn and Yb₂Yn dimers respectively.

Catalytic activities of transferases P, S and N

The specific activities of transferases P and S for a number of substrates were determined and compared with the activities exhibited by the Yb₁Yb₁, Yb₂Yb₂ and YnYn dimers. Two of the substrates tested, DCNB and tPBO, were chosen because the Yb₁ and Yb₂ subunits can be distinguished on the basis of their activities towards these compounds (Mannervik & Jensson, 1982). Table 2 shows that transferase P has a 6-fold greater specific activity for DCNB (the Yb₁-specific substrate) than has transferase S. Conversely, transferase S exhibits a 9-fold greater specific activity for tPBO (the Yb₂-specific substrate) than has transferase P. Although transferase N possessed the highest activity for CDNB of all the enzymes tested, it was not as efficient with DCNB and tPBO as transferases A and D. These data strongly suggest transferases P and S are composed of Yb₁Yn and Yb₂Yn subunits respectively.

The inhibitory effect of bile acids on the catalytic activity of transferases P and S was studied to help elucidate their quaternary structure. Cholate is a more effective inhibitor of the activity of the Yb₁ monomer than of the Yb₂ monomer, whereas chenodeoxycholate and lithocholate 3-sulphate inhibit the Yb₂ monomer more strongly than the Yb₁ monomer. In the present study it was found that cholate inhibits transferase P more readily than transferase S. In contrast, transferase S was inhibited more than transferase P by chenodeoxycholate and lithocholate 3-sulphate, which finding is consistent with the proposed subunit composition of these two enzymes. Transferase N is more resistant to cholate and chenodeoxycholate inhibition than are any of the Yb-containing enzymes studied.

Identification of Yb₁-specific and Yb₂-specific tryptic peptides in digests of transferase P and transferase S

Previous work has shown that the Yb₁ and Yb₂ monomers yield unique tryptic peptides (Beale *et*

Fig. 6. *Electrophoresis of glutathione transferase P* Transferases P, R, L and AA were purified and analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. Transferase K, the acidic YaYa protein in rat liver, which was eluted in the N3 pool from DEAE-cellulose and failed to bind S-hexylglutathione-Sepharose 6B, was purified by glutathione affinity chromatography by using the method of Simons & Vander Jagt (1977). Portions of each were applied to the gel as follows: lane 1, 20 µg of an equimolar mixture of transferase L (YaYa protein) and transferase AA (YcYc protein); lane 2, 5 µg of transferase K; lane 3, 10 µg of transferase P; lane 4, 10 µg of transferase R. The positions of the Ya, Yn, Yb and Yc subunits are indicated. The origin is at the top of the gel (12.5% polyacrylamide), and the samples were run towards the anode.

al., 1983; Hayes, 1983). Tryptic fingerprinting of transferases P and S were therefore performed, to establish whether these proteins contain Yb₁- or Yb₂-specific peptides. Because of paucity of material, peptide maps were constructed by using reverse-phase h.p.l.c. techniques.

The transferases were oxidized with performic acid (Hirs, 1967) and digested with trypsin as described previously (Hayes, 1983). The dried peptides were dissolved in aq. 0.05% (v/v) trifluoroacetic acid and resolved on a Waters

Table 2. Comparison of the catalytic properties of glutathione transferases P and S with other Yb-containing enzymes. For full experimental details see the text.

Transferase	Specific activity ($\mu\text{mol/min per mg of protein}$)			K_m ($\pm$ S.E.M.) for CDNB (mM)
	CDNB	DCNB	tPBO	
A	186	10.7	0.2	0.077 ± 0.004
C	112	4.2	2.2	—
D	34	0.1	3.0	$> 1.8^*$
P	197	11.7	0.2	0.083 ± 0.006
S	139	2.0	1.7	0.206 ± 0.020
N	248	2.3	0.1	—

* Transferase D could not be saturated with CDNB under the experimental conditions owing to the limited solubility of this substrate.

μ Bondapak C-18 (10 μm) column (0.39 cm $\times$ 30.0 cm); this was developed with a 0–60% linear gradient of acetonitrile in water containing 0.05% trifluoroacetate throughout. The chromatogram was developed over a period of 40 min, and the peptides, which were eluted at 1 ml/min, were detected by measuring A_{254} .

Comparisons between the elution profiles of tryptic digests obtained from the YaYc heterodimer and the Yb₁Yb₁, Yb₂Yb₂ and YnYn homodimers permitted the recognition of peptides specific to individual monomers. It was found that repeat analysis of individual digests gave identical results; after alignment of the gradient the chart recordings from different analyses of the same digest were entirely superimposable. The Yb₁-, Yb₂- and Yn-specific peptides were therefore identified by visual inspection (Figs. 7 and 8). Common peptides were identified by applying equimolar mixtures of tryptic digests to the μ -Bondapak column. The existence of the putative common and difference peptides was confirmed by performing three separate digests of each protein, each of which was analysed by h.p.l.c. on at least four occasions.

Fig. 7 shows h.p.l.c. analyses of the tryptic peptides from the Yb₁Yb₁ and Yb₂Yb₂ proteins. A total of 41 peptides were resolved from the digests of the Yb₁Yb₁ dimer and 36 peptides were resolved from the digests of the Yb₂Yb₂ dimer. Comparisons between these profiles demonstrated that 27 of these peptides were co-eluted, that 14 peptides found in Yb₁Yb₁-dimer digests were not found in Yb₂Yb₂-dimer digests and that nine peptides from the Yb₂Yb₂ dimer were not found in Yb₁Yb₁-dimer digests. These results were confirmed by analysing the transferase C (Yb₁Yb₂) digests.

Tryptic digestion of YnYn protein yielded about 45 peptides, which were resolved by h.p.l.c. A comparison between the profiles of the tryptic peptides from YnYn protein and those obtained

from the Yb₁Yb₁ and Yb₂Yb₂ homodimers showed that the Yn monomer appeared to possess peptides common to the Yb₁ and Yb₂ monomers. Fig. 8 shows that ten of the peptides from the YnYn protein were co-eluted with peptides from the Yb₁Yb₁ protein and that ten of the peptides from the YnYn protein were co-eluted with peptides from the Yb₂Yb₂ protein; eight of these were peptides common to both the Yb₁Yb₁ and Yb₂Yb₂ proteins. Larger numbers of hydrophobic peptides were obtained from the Yn monomer than from the Yb₁ and Yb₂ monomers. However, comparisons between the digests of the YnYn protein and transferase B (YaYc) showed that these were unique to the Yn monomer (Fig. 9).

The data presented above demonstrate that about 33 Yn-specific tryptic peptides, 12 Yb₁-specific tryptic peptides and seven Yb₂-specific tryptic peptides can be identified by reverse-phase h.p.l.c. on μ Bondapak columns. This technique was therefore used to determine the quaternary structure of transferases P and S.

About 60 peptides were resolved from the tryptic digests of transferase P. Fig. 10 shows that 11 of the 12 Yb₁-specific peptides were identified in these digests. No Yb₂-specific peptides were recovered. About 50 peptides were resolved from the digests of transferase S. Fig. 11 shows that eight of the nine Yb₂-specific peptides could be identified in the tryptic digests of transferase S, whereas none of the Yb₁-specific peptides was observed. Further, mixtures of digests of transferases P and A or of digests of transferases P and N did not result in the resolution of peaks that were not already observed in the digests of transferase P (Fig. 10). Likewise, the elution of mixtures of the digests of transferases S and D or of digests of transferases S and N from the μ Bondapak column did not result in the identification of peptides not already observed in the digests of transferase S (Fig. 11). These peptide maps therefore provide very strong evidence that

Fig. 7. Resolution and identification of Yb₁- and Yb₂-specific tryptic peptides by using reverse-phase h.p.l.c. The tryptic peptides of performic acid-oxidized transferases A (Yb₁Yb₁) and D (Yb₂Yb₂) were prepared. Portions (300 µg) of digests of the Yb₁Yb₁ protein (a) and of the Yb₂Yb₂ protein (b) were eluted (1 ml/min) from the µBondapak C-18 column (10 µm; 0.39 cm × 30.0 cm) by a 0–60% acetonitrile gradient in aq. 0.05% (v/v) trifluoroacetic acid as described in the text. The time scale is from right to left and the injection point is indicated by an arrow. The continuous straight line represents the developed gradient. The peptides were monitored at 254 nm, and the scale used was 0–0.01 A units. The peptides unique to the Yb₁ subunit are labelled 'A' and those unique to the Yb₂ subunit are labelled 'D'. The letters in parentheses refer to those peptides that were also found in tryptic digests of YnYn protein.

Fig. 8. H.p.l.c. separations of tryptic digests of testicular glutathione transferase N (YnYn protein)

Tryptic digests of transferase N were prepared and resolved by using a μ Bondapak C-18 column as described in the text. Chromatography was performed as indicated in the legend to Fig. 7. The peptides recovered that were common to digests of Yb₁Yb₁ and Yb₂Yb₂ proteins are labelled in accordance with the legend to Fig. 7. Peptides labelled A/D represent those common to Yb₁, Yb₂ and Yn monomers.

Fig. 9. H.p.l.c. separations of tryptic digests of glutathione transferase B (YaYc protein)

Tryptic digests of transferase B were prepared and analysed as described in the text to allow the Yb₁-, Yb₂- and Yn-specific peptides to be compared with those from Ya and Yc subunits, thereby confirming their specificity.

Fig. 10. *H.p.l.c. separations of tryptic digests of glutathione transferase P*
The analysis of tryptic peptides was performed as described in the text. The Yb₁-specific peptides are labelled 'A'.

Fig. 11. *H.p.l.c. separations of tryptic digests of glutathione transferase S*
The analysis of tryptic peptides was performed as described in the text. The Yb₂-specific peptides are labelled 'D'.

Table 3. 'Western blot' analysis of Yb- and Yn-containing glutathione transferases with antiserum raised against the Yb₂ monomer

Equal portions (5 µg) of transferases A, D, N, P and S were subjected to SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper by the method of Towbin *et al.* (1979). The paper was subsequently 'blocked' with gelatin, and after incubation with antiserum against transferase D (raised in New Zealand White rabbits) the cross-reacting proteins were detected by using goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase. Controls were performed to demonstrate that the polypeptides had transferred from the polyacrylamide gel to the nitrocellulose paper.

Transferase	Intensity of peroxidase staining on 'Western blot'	
	10 min	40 min
A	—	—
D	++	+++++
N	—	+
P	—	+
S	++	+++++

transferase P comprises Yb₁ and Yn subunits and that transferase S comprises Yb₂ and Yn subunits.

Cross-reactivity of transferases P and S with antisera raised against Yb₂ polypeptide

During the present study antisera were raised against the Yb₁ and the Yb₂ polypeptides. Although no specific antiserum was obtained against the Yb₁ polypeptide (in two rabbits), antisera were raised in two rabbits against the Yb₂ polypeptide, neither of which cross-reacted with the Yb₁ polypeptide. These antisera both showed about 10% cross-reactivity with the testicular Yn subunit. The antisera against Yb₂ polypeptide strongly reacted with transferase S but not with transferase P. When 'Western blots' of transferases A, D, N, P and S, which had been treated with a primary antibody and a peroxidase-linked second antibody (Bio-Rad Laboratories, Watford, Herts. U.K.), were incubated with 4-chloro-1-naphthol for 10 min (20°C), only transferases D and S showed any peroxidase reaction. Further incubation periods (40 min) resulted in faint reactions being observed with transferases N and P (Table 3).

Discussion

A number of workers have studied the glutathione S-transferases in rat testis (Bass *et al.*, 1977; Dierickx & De Beer, 1981; Eidne & Kirsch, 1982; Guthenberg *et al.*, 1983). However, the purification of a testicular enzyme that was shown to comprise YnYn subunits (transferase N) has not

been described before. The characterization of this enzyme (Table 2) demonstrated that it has a remarkably high specific activity for CDNB, and, although transferase N does not exhibit a particularly high activity for DCNB, it nonetheless possesses a significantly greater activity for DCNB than does transferase D. Transferase N has little activity for tPBO. Bile acids inhibit transferase N in a characteristic fashion; cholate and chenodeoxycholate, the two primary bile acids, serve as poor inhibitors of the CDNB-GSH-conjugating activity of the YnYn dimer whereas lithocholate 3-sulphate is a strong inhibitor.

Since the liver lacks transferase N, the testicular YnYn protein was used for structural studies and its component subunits were found to hybridize with hepatic Yb₁ and Yb₂ subunits. This was a surprising result, since only one YbYn form (transferase S) has been reported to date (Hayes & Chalmers, 1983). Careful examination of liver fractions from chromatofocusing columns that corresponded to the elution positions of the two testicular/hepatic YbYn hybrids revealed the existence of a new, additional, YbYn hybrid (designated transferase P) in rat liver. During the present study a purification scheme has been devised for this enzyme that uses, sequentially, DEAE-cellulose, S-hexylglutathione affinity, chromatofocusing and hydroxyapatite chromatography. The DEAE-cellulose step employed was performed, at pH 8.4, because under these conditions both transferases P and S (the two hepatic YbYn heterodimers) were retained by the exchanger; transferases P and S were recovered respectively in the first and the second peaks of transferase activity that were eluted by the NaCl gradient. This approach enabled the bulk of basic transferase activity, which was eluted in the 'flow-through' fractions from DEAE-cellulose, to be removed during the initial purification step. As DEAE-cellulose did not completely resolve transferases P and S, these conditions resulted in a small portion of transferase S (which served as an invaluable internal standard) being included in the transferase P material applied to the chromatofocusing column (see Fig. 4).

When the purification scheme devised by Habig *et al.* (1974) is employed, transferase P is eluted in the 'flow-through' fractions from DEAE-cellulose and is found to be eluted from CM-cellulose with transferase C. Although not identified as such, the Yn subunit can be clearly recognized in the electrophoretic analysis of the transferase-C-containing eluate from CM-cellulose described by Sheehan & Mantle (1984). The use of hydroxyapatite after CM-cellulose resolves transferases C and P (Fig. 5).

Transferases P and S are catalytically distinct

and can be distinguished by their activities with tPBO and DCNB. Further, the substrate specificities of transferases P and S suggest that these enzymes comprise Yb₁ and Yn subunits and Yb₂ and Yn subunits respectively. A comparison of the inhibition of transferases A, D, N, P and S by various bile acids is also consistent with transferases P and S containing Yb₁ and Yn subunits and Yb₂ and Yn subunits respectively.

The use of 'Western blotting' techniques (Towbin *et al.*, 1979; Burnette, 1981) provided strong immunological evidence that transferase S contains a Yb₂ subunit.

Peptide-mapping experiments demonstrated that transferase P represents the Yb₁Yn dimer and transferase S the Yb₂Yn dimer. The Yb₁-specific tryptic peptides were only recovered from digests of transferase P, and Yb₂-specific peptides were only recovered from digests of transferase S. Reversible denaturation of hepatic transferase S unequivocally identified the presence of the Yb₂ subunit by its elution position from chromatofocusing columns and by the fact it lacked DCNB-GSH-conjugating activity under assay conditions that permitted the activity of the Yn monomer with DCNB to be detected.

The peptide-mapping and hybridization data suggest that the hepatic and testicular Yn polypeptides are probably identical. All the tryptic peptides from testis transferase N resolved by h.p.l.c. were present in the digests of hepatic transferase S and transferase P. In addition, the facts that the YnYn protein obtained after reversible denaturation of hepatic transferase S and testis transferase N are eluted at identical positions from chromatofocusing columns and that both possess DCNB-GSH-conjugating activity supports this contention.

Previous work has demonstrated that the Yb₁Yb₁ dimer exists in three forms (A₁, A₂ and A₃), which are thought to arise as the result of autooxidation of a single residue in the Yb₁ polypeptide (Hayes & Clarkson, 1982; Friedberg *et al.*, 1983). It is therefore possible that, since transferase P contains the Yb₁ subunit, a reduced and an oxidized form of this enzyme exist. No multiplicity of transferase P was observed in the present study. However, after S-hexylglutathione affinity chromatography of testis cytosol, multiple forms of YnYn protein were resolved by chromatofocusing (these were eluted at pH 6.6, pH 6.4 and pH 6.25) when the dialysis time, to remove the S-hexylglutathione, before the chromatofocusing step was shortened from 16 h to 9 h. Subsequent analysis of the YnYn forms showed they were each eluted at identical positions from hydroxyapatite columns, that they were catalytically indistinguishable and that they yielded identical peptide maps.

When the pre-chromatofocusing dialysis time was increased to 16–20 h transferase N was eluted at pH 6.6 as a single peak from the PBE 94 column, suggesting that the multiple forms arose from an S-hexylglutathione interaction with the Yn monomer.

The Yb₁ and Yb₂ subunits appear to have similar affinities for the Yn subunit, as reversible denaturation of mixtures containing equimolar concentrations of the three polypeptides resulted in the formation of both Yb₁Yn and Yb₂Yn dimers. As significant amounts of YnYn protein were recovered in the hybridization experiments, it is concluded that the YnYn homodimer is as stable as the heterodimers to which it gives rise during the re-association process. Since it is likely that re-association involves self-recognition between the interaction sites of hybridizing subunits, the Yb₁, Yb₂ and Yn polypeptides can be expected to possess extensive homology around these areas. It should therefore be noted that the peptide maps showed that the Yb₁, Yb₂ and Yn polypeptides shared a considerable number of common tryptic peptides. If the Yb₁, Yb₂ and Yn polypeptides have been formed from a single ancestral gene, then the interaction site between subunits is likely to be highly conserved. The fact that both Yn and Yb₁ possess significant activity for DCNB and little activity for tPBO whereas, conversely, Yb₂ possesses no activity for DCNB and high activity for tPBO suggests that the active centres of the Yn and Yb₁ subunits may be similar. However, marked differences in the effects of bile acids on transferases A (Yb₁Yb₁) and N (YnYn) exist. Transferase N is much less susceptible to cholate inhibition than is transferase A; the I₅₀ values exhibited by these enzymes for cholate differ by a factor of 5-fold. In contrast, transferase N is inhibited more by lithocholate 3-sulphate than is transferase A.

The inducibility of transferase subunits by various xenobiotics is interesting, since Sheehan *et al.* (1984) have reported that, in contrast with the liver, the concentration of Yb₂, and not Yb₁, monomers in rat testis is increased by phenobarbitone treatment. The inducibility of the Yn monomer has not been investigated, nor is it known what effect induction of Yb₁ or Yb₂ monomers has on the concentrations of Yb₁Yn or Yb₂Yn protein.

The reason for the dimeric structure of the transferases is not known. Since Mannervik and his colleagues have shown that individual transferase subunits function independently, there appears to be no reason, from a catalytic viewpoint, why the transferases should comprise more than one polypeptide (Jakobson *et al.*, 1977; Mannervik & Jensson, 1982). It is possible that the transferases possess an alternative, hitherto un-

identified, function that requires a dimeric quaternary structure. Alternatively, interactions may occur between certain subunits that enhance the catalytic efficiency of the resulting hybrid enzymes for particular substrates. It is also conceivable that the formation of hybrid enzymes represents a mechanism that enables a particular monomer to occupy a niche to which it could not otherwise gain access, as it is known that significant differences exist in the cellular locations of certain transferases (Redick *et al.*, 1982).

Although it is now well established that the Yb₁ subunit can hybridize with the Yb₂ subunit, the present study has shown for the first time that the Yb₁ subunit can form hybrids with a second, alternative, subunit (i.e. Yn). The existence of the Yb₁Yn dimer is interesting because this represents the first demonstration of the Yb₁ subunit associating with a monomer of a different molecular mass. Indeed, since the Yb₂ subunit can also hybridize with the Yn subunit it is apparent that combinations of the Yb₁, Yb₂ and Yn subunits result in the formation of a total of six isoenzymes.

It should be noted that, in contrast with the findings obtained by previous workers, antiserum has been obtained that can discriminate between the two hepatic YbYb homodimers. This antiserum should be useful in helping elucidate mechanisms that control the expression of hepatic Yb₂ subunit.

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