Purification and physical characterization of glutathione *S*-transferase K

Differential use of *S*-hexylglutathione and glutathione affinity matrices to isolate a novel glutathione *S*-transferase from rat liver

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A novel hepatic enzyme, glutathione *S*-transferase K, is described that, unlike previously characterized transferases, possesses little affinity for *S*-hexylglutathione–Sephadex 6B but can be isolated because it binds to a glutathione affinity matrix. A purification scheme for this new enzyme was devised, with the use of DEAE-cellulose, *S*-hexylglutathione–Sephadex 6B, glutathione–Sephadex 6B and hydroxyapatite chromatography. The final hydroxyapatite step results in the elution of three chromatographically interconvertible forms, K₁, K₂ and K₃. The purified protein has an isoelectric point of 6.1 and comprises subunits that are designated Yk (*M_r* 25000); during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, it migrates marginally faster than the Ya subunit but slower than the pulmonary Yf monomer (*M_r* 24500). Transferase K displays catalytic, immunochemical and physical properties that are distinct from those of other liver transferases. Tryptic peptide maps suggest that transferase K is a homodimer, or comprises closely homologous subunits. The tryptic fingerprints also demonstrate that, although transferase K is structurally separate from previously described hepatic forms, a limited sequence homology exists between the Yk, Ya and Yc polypeptides. These structural data are in accord with the immunochemical results presented in the accompanying paper [Hayes & Mantle (1986) *Biochem. J.* 233, 779–788].

INTRODUCTION

Glutathione *S*-transferases catalyse the conjugation of GSH with a wide range of electrophiles and represent a major drug-metabolizing system (Chasseaud, 1979; Habig & Jakoby, 1981).

The transferases are dimeric, and the large number of isoenzymes that occur in man and the rat arise from the existence of multiple genes and the ability of certain subunits to form hybrids (Hayes *et al.*, 1981; Beale *et al.*, 1982, 1983; Boyer *et al.*, 1983; Frey *et al.*, 1983; Hayes, 1983, 1984; Stockman *et al.*, 1985). In the rat, the nomenclature that is most widely used is that which defines the transferase monomers by their apparent differences in size when examined by discontinuous SDS/polyacrylamide-gel electrophoresis: Ya (*M_r* 25500), Yn (*M_r* 26500), Yb₁ (*M_r* 27000), Yb₂ (*M_r* 27000) and Yc (*M_r* 28500) (Beale *et al.*, 1983; Hayes, 1983; Hayes & Chalmers, 1983).

In the present study I describe a novel enzyme, transferase K, prepared from rat liver. It contains subunits that appear to be marginally smaller than the Ya subunit when examined by SDS/polyacrylamide-gel electrophoresis. Transferase K does not bind to *S*-hexylglutathione–Sephadex 6B (prepared by the method of Mannervik & Guthenberg, 1981), but can be isolated by using the glutathione affinity matrix described by Simons & Vander Jagt (1977). The structural and catalytic properties of this new enzyme are described below.

EXPERIMENTAL

Materials

The chemicals used were all of analytical grade and were obtained commercially unless otherwise stated.

Buffers

These were all prepared at 20 °C. The pH values of the buffers used for enzyme purification were measured at 4 °C, and the values quoted in the text are those found at this temperature.

Animals

Livers from male Wistar rats (250–400 g) fed *ad libitum* were used as enzyme source. The livers were normally stored at –85 °C for 1–10 days before use.

Analytical methods

Enzyme assays were performed at 37 °C with either a centrifugal analyser (Hayes & Clarkson, 1982) or manual methods (Habig & Jakoby, 1981).

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970), with either a Raven IN/96 apparatus (Raven Scientific, Haverhill, Suffolk, U.K.) or an LKB 2001 vertical electrophoresis unit (LKB Instruments, Selsdon, Surrey, U.K.).

Analytical isoelectric focusing in thin-layer 6% (w/v) polyacrylamide gel was performed in an LKB Multiphor apparatus as described by the manufacturer. The protein

pI standards used to calibrate the gel were from Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K.

The amino acid compositions were kindly determined by Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, Scotland, U.K.

Purification of glutathione *S*-transferase K

Frozen rat liver (250–350 g) was allowed to thaw at room temperature before being blended in 2 vol. of ice-cold 10 mM-Tris/HCl buffer, pH 8.1. All subsequent steps were performed at 4 °C. The resulting material was centrifuged (15000 *g* for 1 h) and, after filtration through a plug of glass-wool, was applied directly to DEAE-cellulose DE52 equilibrated with 10 mM-Tris/HCl buffer, pH 8.1. Normally either two 3.2 cm × 85 cm columns or two 4.4 cm × 85 cm columns were employed. These were used in series, and the anion-exchanger was developed with a 0–125 mM-NaCl gradient formed by a 350 ml constant-volume mixing reservoir. The fractions containing the last significant peak of transferase activity that was eluted in the Na⁺ concentration range 60–85 mM [this has previously been designated N3 (Hayes, 1983)] were combined.

The N3 pool was applied directly to a 2.2 cm × 20 cm column of *S*-hexylglutathione-Sepharose 6B equilibrated with 20 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl. The transferase activity that was not retained by the *S*-hexylglutathione-Sepharose 6B affinity gel, and that was eluted in the flow-through fractions, was combined and applied directly to a 2.2 cm × 20 cm column of a glutathione-Sepharose 6B affinity matrix prepared as described by Simons & Vander Jagt (1977). This affinity gel was equilibrated with 100 mM-Tris containing 100 mM-Na₂HPO₄ adjusted to pH 9.0 with HCl. The transferase activity that was adsorbed on the column was washed (30 ml/h) with at least 200 ml of the same buffer before being eluted with a 50 mM solution of GSH in the running buffer (this had a pH of approx. 8.1). The material eluted by GSH was dialysed for 18 h against three changes, each of 2 litres, of 10 mM-sodium phosphate buffer, pH 6.7, containing 2 mM-2-mercaptoethanol. After dialysis, the transferase was finally purified on 1.6 cm × 19 cm columns of hydroxyapatite, eluted (25 ml/h) at pH 6.7 and developed with a 10–175 mM-sodium phosphate gradient, formed by a 350 ml constant-volume mixing reservoir, that contained 2 mM-2-mercaptoethanol throughout.

Purification of other glutathione *S*-transferases

In addition to glutathione *S*-transferase K, other rat hepatic and testicular transferases were prepared for comparative purposes. These were isolated as described previously (Hayes, 1983, 1984; Hayes & Chalmers, 1983). Transferase λ (YfYf) was used as an electrophoretic marker and was purified from human lung; it was purified by *S*-hexylglutathione affinity chromatography and eluted at pH 4.8 from PBE 94 chromatofocusing columns (1.6 cm × 35 cm) developed by pH 6.0–pH 4.0 gradients (J. D. Hayes & J. Chalmers, unpublished work).

Tryptic peptide maps

These were constructed by the methods of Ambler (1963). Whatman 3MM paper was used throughout. The Wondermix cocktail of amino acids, used to standardize chromatograms, has been described previously (Ambler

& Brown, 1967). Portions (4–12 mg) of performic acid-oxidized glutathione *S*-transferase were digested for 8–16 h at 37 °C with trypsin (transferase/proteinase ratio 40:1, w/w). After freeze-drying and dissolution, the digests were resolved by high-voltage paper electrophoresis at pH 6.5 into the acidic, the neutral and the basic peptides. This step served as the first dimension for the maps of the acidic and the basic peptides; the neutral peptides (which migrated cathodally as a single spot at pH 6.5) were cut out of the paper and subsequently run out in their first dimension by high-voltage electrophoresis at pH 3.5.

Typically, high-voltage paper electrophoresis at pH 6.5 was performed until the red Pentel-pen marker ink migrated anodally about 12 cm from the origin (during which time the aspartate in Wondermix migrated anodally 13.5 cm and lysine migrated cathodally 14.0 cm). Likewise, high-voltage paper electrophoresis at pH 3.5 was performed until the Methyl Green dye migrated cathodally about 30 cm (this dye roughly migrated with the lysine and histidine in Wondermix). The acidic peptides were resolved in the second dimension by high-voltage paper electrophoresis at pH 3.5. In contrast, the second dimension of both the neutral and the basic peptide maps comprised descending paper chromatography, performed for 16–19 h, with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent.

Immuno-blotting

The preparation of antisera and the immuno-blotting method is described elsewhere (Hayes & Mantle, 1986b).

RESULTS

Identification of transferase K

The neutral glutathione *S*-transferases in rat liver are resolved into three peaks of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity by DEAE-cellulose chromatography (Fig. 1). These peaks (previously referred to by their order of elution as N1, N2 and N3) contain transferases that can be purified by *S*-hexylglutathione affinity chromatography and that have been shown to comprise combinations of Yb- and Yn-type subunits (Hayes, 1983, 1984; Hayes & Chalmers, 1983). No neutral or acidic transferases containing Ya- or Yc-type monomers have been isolated from rat liver.

Electrophoretic analysis of fractions from DEAE-cellulose containing neutral transferase activity demonstrated that, in addition to the Yb polypeptides, a monomer was present in the N3 pool with a mobility similar to the basic Ya subunit (Fig. 2a). Attempts were therefore made to isolate this polypeptide, with the use of standard affinity purification steps to establish whether it possessed transferase activity.

S-Hexylglutathione affinity chromatography of the N3 pool resulted in the major portion of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity being retained by the matrix, but consistently a significant amount of this activity (typically about 15% of the total N3 pool) was eluted in the flow-through fractions from this affinity gel. The material that was specifically adsorbed on *S*-hexylglutathione-Sepharose consisted mainly of Yb-type polypeptides. Application of the N3 material that did not bind to the *S*-hexylglutathione-agarose gel to a 2.2 cm × 20 cm column of glutathione-Sepharose 6B

Fig. 1. Resolution of various hepatic neutral/acidic glutathione *S*-transferase activities by DEAE-cellulose

The 15000 *g* supernatant (465 ml) from 225 g of rat liver was eluted (31 ml/h) from DEAE-cellulose (bed volume 1.8 litres). The column, which was equilibrated with 10 mM-Tris/HCl buffer pH 8.1, was developed with a 0–125 mM-NaCl gradient, formed by using a 350 ml constant-volume mixing reservoir, to elute the neutral/acidic enzymes. Fractions (10.4 ml) were collected and transferase activity was measured with 1-chloro-2,4-dinitrobenzene ($\blacktriangle$), 1,2-dichloro-4-nitrobenzene ($\circ$), 1-menaphthyl sulphate ($\triangle$), 1,2-epoxy-3-(*p*-nitrophenoxy)propane ($\square$) and *trans*-4-phenylbut-3-en-2-one ($\bullet$) as substrates. The Na^+ concentrations ($\blacksquare$) were determined by flame photometry.

resulted in the binding and subsequent specific elution of transferase activity towards 1-chloro-2,4-dinitrobenzene. In contrast, the menaphthyl sulphate–GSH-conjugating activity failed to bind to the second affinity gel, suggesting that the glutathione affinity step had resulted in the isolation of an enzyme distinct from transferase M [see Gillham (1973) for details of transferase M]. Fig. 2 shows that the glutathione–Sephacrose 6B column selected a subunit that was present in only minor amounts in the N3 material bound by the *S*-hexylglutathione affinity column. It is therefore clear that the glutathione affinity step had facilitated the isolation of a new form of enzyme, henceforth referred to as glutathione *S*-transferase K. Sequential elution of the N1 and N2 fractions from the same columns did not yield other enzymes with similar chromatographic or electrophoretic properties.

Purification of multiple forms of transferase K

Electrophoresis demonstrated the presence of minor contaminants in the transferase K preparation obtained from the glutathione–Sephacrose 6B column (Fig. 2b). These impurities were removed by hydroxyapatite chromatography, a step that resolved transferase K into three peaks of 1-chloro-2,4-dinitrobenzene–GSH-conjugating activity; these were eluted at Na^+ concentrations of 115, 135 and 151 mM and were designated K_1 , K_2 and

K_3 respectively (Fig. 3). It is likely that these three forms are interconvertible, since, when 2 mM-2-mercaptoethanol was included in the running and limit buffers, transferase activity was eluted as a single, though asymmetric, peak at an Na^+ concentration of about 120 mM (results not shown).

The unusual chromatographic properties of transferase K are emphasized by the fact that transferases K_1 and K_2 possess less affinity for hydroxyapatite than do other transferases. Previous work, with identical columns, has demonstrated that the group I transferases (Ya- and Yc-containing forms) show less affinity than do the group II transferases (Yb- and Yn-containing forms) for hydroxyapatite (Hayes, 1984). A comparison between the elution properties of transferases K_1 , K_2 , K_3 , L (YaYa) and AA (YcYc) from hydroxyapatite is included in Fig. 3. The results of the purification of transferase K are summarized in Table 1.

Comparison of the electrophoretic properties of transferase K with those of other glutathione *S*-transferases

After SDS/polyacrylamide-gel electrophoresis transferases K_1 , K_2 and K_3 were indistinguishable; they each possessed an apparent M_r of 25000. This calculation is based on an assumed M_r value of 25500 for Ya, derived from the deduced amino acid sequences of cDNA probes

Fig. 2. Electrophoresis of fractions obtained during the purification of glutathione S-transferase K

Transferase K eluted from DEAE-cellulose in the N3 pool (fractions 221–234). Panel (a) shows the result of SDS/polyacrylamide-gel electrophoresis of: lane 1, a mixture of Ya and Yc subunits; lane 2, fraction 201 from DEAE-cellulose; lane 3, fraction 212 from DEAE-cellulose; lane 4, fraction 224 from DEAE-cellulose. The arrow shows the polypeptide in fraction 224 that possesses a mobility similar to that of the basic Ya subunit. Transferase K was identified on the basis that it bound to *S*-hexylglutathione-Sepharose 6B poorly but was retained by glutathione-Sepharose 6B. Transferase K was therefore purified from the flow-through fractions of a *S*-hexylglutathione affinity column by glutathione affinity chromatography followed by hydroxyapatite chromatography. The purification steps analysed by SDS/polyacrylamide-gel electrophoresis are shown in panel (b) as follows: lane 1, 10 μ g of transferase B; lane 2, 15 μ g of the transferases in the N3 pool purified by *S*-hexylglutathione affinity chromatography; lane 3, 15 μ g of the N3 material that was obtained from the flow-through fractions of *S*-hexylglutathione-Sepharose 6B and specifically bound to glutathione-Sepharose 6B; lane 4, 15 μ g of purified transferase K after hydroxyapatite chromatography; lane 5, 10 μ g of transferase B.

Table 1. Purification of glutathione S-transferase K

For full experimental details see the text. The neutral transferases were resolved by DEAE-cellulose chromatography into three peaks of activity (N1, N2 and N3), which were eluted at Na⁺ concentrations 25–40 mM, 42–58 mM and 60–85 mM respectively. N2 and N3 pools were combined before the affinity steps to maximize the recovery of transferase K and demonstrate the distinct chromatographic behaviour of transferase M (the form that is specific for menaphthyl sulphate). Abbreviations: MS, 1-menaphthyl sulphate; ENPOP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene.

Fraction	Volume (ml)	Protein (A_{280} units)	Total activity (μ mol/min)			
			With MS	With ENPOP	With DCNB	With CDNB
1. Total neutral transferases (N1 + N2 + N3)	400	3432	133	1740	272	13270
2. Total N2 + N3 pool	200	2442	142	240	80	6170
3. N2 + N3 purified by <i>S</i> -hexylglutathione-Sepharose	19	63	0	29	62	3340
N2 + N3 flow-through from <i>S</i> -hexylglutathione-Sepharose	320	2176	125	200	0	530
4. N2 + N3 purified by glutathione-Sepharose	18	30	0	0	0	348
N2 + N3 flow-through from glutathione-Sepharose	320	1845	96	192	0	114
5. Hydroxyapatite chromatography of N2 + N3 purified by glutathione-Sepharose:						
K ₁	40	6	0	0	0	90
K ₂	40	6.5	0	0	0	98
K ₃	45	1.5	0	0	0	22

Fig. 3. Resolution of glutathione *S*-transferases by hydroxyapatite chromatography

Partially purified transferase K, eluted from glutathione–Sephacrose 6B, was dialysed against 10 mM-sodium phosphate buffer, pH 6.7, containing 0.5 mM-2-mercaptoethanol, and applied to hydroxyapatite columns (1.6 cm × 19.0 cm) equilibrated with the same buffer. The columns were eluted at 22.5 ml/h and were developed with a 10–175 mM-sodium phosphate gradient, formed by using a 350 ml constant-volume mixing reservoir, containing 0.5 mM-2-mercaptoethanol throughout. Partially purified transferases L and AA, obtained after DEAE-cellulose chromatography, $(\text{NH}_4)_2\text{SO}_4$ precipitation and CM-cellulose chromatography (Hayes, 1983), were run in parallel during the preparation of different transferase K batches and are included for comparison. Fractions (4.5 ml) were collected and the Na^+ concentration (●), absorbance at 280 nm (○) and transferase activity with 1-chloro-2,4-dinitrobenzene (▲) were measured. Panels (a), (b) and (c) represent the hydroxyapatite elution profiles from transferases K, L and AA. The horizontal bars indicate the fractions that were combined and, in the case of transferase K, represent the final purified products. In the case of transferases L and AA, the enzymes were subjected to *S*-hexylglutathione affinity chromatography before peptide mapping experiments (Figs. 5–7).

encoding the Ya subunit (Lai *et al.*, 1984; Pickett *et al.*, 1984). An electrophoretic comparison (Fig. 4) of these forms with transferases L (YaYa) and AA (YcYc), as well as enzymes purified from rat spleen and rat lung (both of which contain Yf polypeptides), demonstrated that transferase K migrated between the Ya and Yf subunits. There are therefore two transferase monomers with a faster electrophoretic mobility than the Ya subunit, referred to as Yk (obtained from transferase K) and Yf (present in rat lung and rat spleen). Fig. 4 shows that rat spleen contains both Yf and Yk monomers and that these can be distinguished by their chromatographic behaviour on affinity columns. This gel also demonstrates that the

Yf monomer in rat lung co-migrates during electrophoresis with transferase λ from human lung.

The two major forms of transferase K, K_1 and K_2 , possessed identical isoelectric points, pI 6.1. The pI of K_3 was not determined, owing to lack of material.

Catalytic properties of transferase K

The activities of transferases K_1 , K_2 and K_3 for a variety of substrates were compared and contrasted with those of other enzymes (Table 2). These comparisons showed that transferases K_1 , K_2 and K_3 possess very similar substrate specificities, but are distinct from the activities expressed by other transferases. Transferase K

Fig. 4. Electrophoretic comparison of glutathione *S*-transferase K with Yf-containing and Ya-containing enzyme preparations

SDS/polyacrylamide-gel electrophoresis was performed in a 12% resolving gel and stained with Coomassie Blue R250. Rat lung and spleen transferases were separately purified from 100000 *g* supernatants by *S*-hexylglutathione affinity chromatography. Spleen transferases that did not bind to *S*-hexylglutathione-Sepharose 6B were isolated by glutathione affinity chromatography. Human transferase λ , which was used as the Yf marker, was purified as described in the text. Transferase L, used as the Ya marker, was from the hydroxyapatite pool shown in Fig. 3 and had not been subjected to affinity chromatography. Samples, which were all from rat tissues (with the exception of transferase λ), were applied as follows: lane 1, transferase L (YaYa); lane 2, rat lung transferases; lane 3, transferase λ (YfYf) from human lung; lane 4, transferase K (YkYk); lane 5, transferase L; lane 6, transferase K; lane 7, partially purified transferase B; lane 8, spleen transferases purified by *S*-hexylglutathione-Sepharose 6B chromatography; lane 9, additional spleen transferases that did not bind to *S*-hexylglutathione-Sepharose 6B and that were purified by glutathione-Sepharose 6B chromatography.

Table 2. Comparison of the catalytic properties of glutathione *S*-transferases K₁, K₂ and K₃ with those of other enzymes

For full experimental details see the text. All analyses were performed at 37 °C. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; *p*NBC, *p*-nitrobenzyl chloride; *p*NPA, *p*-nitrophenyl acetate; DCNB, 1,2-dichloro-4-nitrobenzene; BSP, bromosulphophthalein; *t*PBO, *trans*-4-phenylbut-3-en-2-one. A value of zero denotes that, after the addition of 50 μ g of protein, the rate observed was not significantly different from the blank (no enzyme added) rate. Table 1 shows that transferase K also lacks activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 1-menaphthyl sulphate.

Transferase	Group	Subunits	Specific activity (μ mol/min per mg of protein)						
			With CDNB	With EA	With <i>p</i> NBC	With <i>p</i> NPA	With DCNB	With BSP	With <i>t</i> PBO
K ₁	I	YkYk	15	9.5	1.1	0.09	0	0	0
K ₂	I	YkYk	15	9.3	1.1	0.09	0	0	0
K ₃	I	YkYk	16	10.9	1.2	0.05	0	0	0
F	I	YaYa	65	0.4	1.1	0.56	0.09	0	0
L	I	YaYa	96	0.4	1.5	1.32	0.11	0	0
AA	I	YcYc	51	3.5	0.3	0.58	0.06	0	0
A	II	Yb ₁ Yb ₁	186	0	14.0	0.18	10.7	1.57	0.2
D	II	Yb ₂ Yb ₂	34	0.7	14.4	0.23	0.1	0	3.0
N	II	YnYn	248	0	1.7	0.05	2.3	0	0.1

exhibits a very low specific activity for 1-chloro-2,4-dinitrobenzene and a high activity for ethacrynic acid, and thus a characteristic ratio of activities for these two substrates. In common with the group I enzymes, transferase K possesses little activity with 1,2-dichloro-4-nitrobenzene, bromosulphophthalein or *trans*-4-

phenylbut-3-en-2-one (electrophiles that appear to be specific for the group II enzymes).

Structural characterization of transferase K

Antisera were raised against transferase K (the form eluted from hydroxyapatite under reducing conditions at

Table 3. Amino acid composition of glutathione *S*-transferase K, compared with those of group I and II enzymes

Samples (about 1 mg of protein) were hydrolysed in 6M-HCl for 18 h. The compositions were not corrected for the increased or decreased recoveries of serine, threonine, valine and isoleucine. Results are normalized on a total recovery of 1 μ mol of amino acid. The values in parentheses are estimates of the number of residues per dimer, assuming the subunit M_r values shown.

Amino acid	Transferase Subunit composition† Subunit M_r	Amino acid composition (nmol recovered from 18 h hydrolysate)				
		K YkYk 25000	F YaYa 25500	L YaYa 25500	C* Yb ₁ Yb ₂ 27000	AA YcYc 28500
Lys		87.3 (38)	99.2 (44)	110.0 (48)	106.1 (49)	88.2 (43)
His		8.8 (4)	11.0 (5)	11.5 (5)	23.7 (11)	17.4 (9)
Arg		58.8 (25)	55.9 (25)	57.6 (25)	62.9 (29)	69.2 (34)
Asx		70.3 (30)	97.7 (43)	98.9 (44)	113.6 (53)	93.3 (46)
Thr		35.7 (15)	32.9 (14)	32.2 (14)	31.8 (15)	20.8 (10)
Ser		35.6 (15)	40.2 (18)	40.3 (18)	51.4 (24)	38.9 (19)
Glx		151.4 (65)	144.0 (63)	134.7 (59)	108.9 (51)	131.4 (64)
Pro		49.6 (21)	40.9 (18)	39.3 (17)	54.1 (25)	49.7 (24)
Gly		65.8 (28)	63.6 (28)	55.9 (25)	51.4 (24)	58.4 (29)
Ala		72.4 (31)	73.4 (32)	74.9 (33)	49.4 (23)	79.5 (39)
Val		65.7 (28)	46.7 (20)	46.5 (20)	25.7 (12)	75.5 (37)
Met		33.3 (14)	34.3 (15)	33.5 (15)	34.5 (16)	25.9 (13)
Ile		46.4 (20)	49.4 (22)	50.0 (22)	51.4 (24)	37.2 (18)
Leu		128.5 (55)	133.2 (59)	134.2 (59)	120.3 (56)	125.7 (62)
Tyr		41.0 (18)	34.3 (15)	34.8 (15)	58.8 (27)	48.9 (24)
Phe		54.6 (23)	43.2 (19)	45.8 (20)	56.1 (26)	39.9 (20)

* Data for transferase C (Yb₁Yb₂) are from Hayes (1983); determinations were on that occasion also performed by Dr. R. P. Ambler.

† Since the existence of Ya₁ and Ya₂ has not yet been demonstrated, transferases F and L are both designated YaYa.

Fig. 5. Two-dimensional map of the acidic peptides from glutathione *S*-transferases K (YkYk), L (YaYa) and AA (YcYc)

Tryptic digests of the oxidized enzymes were prepared as described in the text. Maps of the acidic peptides were prepared by high-voltage paper electrophoresis (H.v.p.e.) at pH 6.5 in the first dimension and at pH 3.5 in the second dimension. The origin (O) and the positions of migration of alanine (A), aspartic acid (D), glycine (G), glutamic acid (E), lysine (K), arginine (R), valine (V), Xylene CFF (X) and the red Pentel-pen marker (RP) are shown. The peptide that appears to be common to all three proteins is represented by the blackened spots, and the peptide common to Ya and Yc by hatched spots.

Fig. 6. Two-dimensional map of the neutral peptides from glutathione *S*-transferases K (YkYk), L (YaYa) and AA (YcYc)

Portions (4 mg) of the tryptic digests were subjected to high-voltage electrophoresis at pH 6.5, and the neutral peptides, which migrated cathodally about 2–4 cm, were isolated. These were cut out and separated in the first dimension by high-voltage paper electrophoresis (H.v.p.e.) at pH 3.5 and in the second dimension by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). The positions of migration of alanine (A), glycine (G), leucine (L), Xylene CFF (X), valine (V) and proline (P) are shown. The peptides that appear to be common to all three proteins are represented by the blackened spots, those that appear to be common to transferases L (the Ya dimer) and AA (the Yc dimer) by the hatched spots, and those that appear to be common to transferases K and L (YaYa) by cross-hatched spots.

an Na^+ concentration of 120 mM was used as the immunogen) and their immunochemical properties investigated. The results are described in detail in the accompanying paper (Hayes & Mantle, 1986b). Briefly, however, under optimized assay conditions transferase K (YkYk) was found to be immunochemically distinct, but the anti-transferase K) IgG showed approx. 15% cross-reactivity with Ya and Yc subunits when a 5-fold excess of antiserum was employed. There was no cross-reactivity under any condition between transferase K and antisera raised against either individual group II enzymes (Yb₁Yb₁, Yb₂Yb₂ or YnYn forms) or transferase λ (a form comprising Yf-type subunits). Likewise, anti-(transferase K) IgG did not cross-react with the group II enzymes or transferase λ (Hayes & Mantle, 1986b).

The amino acid composition of transferase K appears to be distinct (Table 3). For example, the valine and aspartic acid/asparagine contents of transferase K differ significantly from those of the Ya-, Yb- and Yc-containing enzymes. However, similarities between transferases K, F (YaYa) and L (YaYa) exist in the number of histidine, arginine and alanine residues represented, a feature that is not apparent when transferase K is compared with transferases C (Yb₁Yb₂) and AA (YcYc).

The peptide map of transferase K was constructed and compared with maps obtained from transferases F (YaYa), L (YaYa) and AA (YcYc): (1) to determine whether transferase K is a homodimer; (2) to establish the structural relationship between transferase K and the group I enzymes, since these proteins display some immunochemical cross-reactivity (Hayes & Mantle, 1986b). Marked differences exist between the fingerprints

of soluble peptides obtained from tryptic digests of transferases K, L and AA (Figs. 5, 6 and 7), indicating that the Yk, Ya and Yc subunits that these enzymes comprise are distinct polypeptides. The fingerprints of the two YaYa-containing enzymes, transferases F and L, were indistinguishable (results not shown).

Transferase K yielded a total of 30 tryptic peptides (six acidic, nine neutral and 15 basic). Transferases F and L both yielded 34 tryptic peptides (five acidic, 12 neutral and 17 basic). Transferase AA yielded 29 peptides (five acidic, 11 neutral and 13 basic). A highly acidic tryptic peptide, which migrated anodally ahead of the red Pentel-pen marker dye and appeared to be common to all four enzymes, is not shown in the acidic peptide map (Fig. 5) because of the physical constraints in graphically reproducing and constructing the fingerprints.

The fact that digests of transferase K yielded only 30 soluble tryptic peptides suggests that it is more likely to be a homodimer rather than a heterodimer, since the amino acid composition of this enzyme shows there are approx. 63 arginine + lysine residues per dimer.

A total of seven peptides (two acidic, one neutral and four basic) appear to be common to Yk, Ya and Yc subunits. Three additional peptides (one neutral and two basic) appear to be common to Yk and Ya subunits. No peptides were identified that were present in digests of both Yk and Yc subunits but not in digests of Ya subunits.

DISCUSSION

A purification scheme was devised for a hitherto unknown glutathione *S*-transferase, transferase K

Fig. 7. Two-dimensional map of the neutral peptides from glutathione *S*-transferases K (YkYk), L (YaYa) and AA (YcYc)

Tryptic digests of transferases K, L and AA were separated by high-voltage paper electrophoresis (H.v.p.e.) at pH 6.5. In the second dimension the basic peptides were resolved by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent for 16 h. The origin (O) and the position where the paper was cut after electrophoresis, to remove the neutral peptides (indicated by arrows), are shown. The positions of migration of the neutral amino acids (N), lysine (K), arginine (R), histidine (H), leucine (L), Xylene CFF (X), valine (V) and proline (P) are indicated. The significance of blackened, hatched and cross-hatched spots is indicated in the legend to Fig. 6.

(YkYk), prepared from rat liver. This enzyme has two features that have probably contributed to the fact that it has previously gone undetected. Firstly, it displays a very low specific activity for 1-chloro-2,4-dinitrobenzene. Secondly, it possesses a low affinity for the *S*-hexylglutathione-Sepharose 6B matrix, a gel that is widely used for purification of glutathione *S*-transferases.

Transferase K is catalytically distinct from previously described hepatic enzymes. It can be distinguished by its high specific activity for ethacrynic acid and relatively low specific activities for 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride and *p*-nitrophenyl acetate (Table 2). Further, the characteristic inhibitory effects of bile acids on individual transferase subunits enables these ligands to be employed as an aid to enzyme identification (Hayes & Mantle, 1986a). For example, at pH 6.0 and 6.5, cholate (a trihydroxy primary bile acid) inhibits transferase K more than it does any other hepatic enzyme.

The final hydroxyapatite purification step resulted in transferase K being resolved into three peaks, K_1 , K_2 and K_3 , all having very similar catalytic activities. The chromatographic behaviour of these three peaks on this column suggested that the K_2 and K_3 forms were being converted into the K_1 form in the presence of 2-mercaptoethanol. This possibility is supported by the fact that forms K_1 and K_2 yielded identical tryptic fingerprints (paucity of material prevented a structural examination of K_3).

Transferase K comprises subunits (Yk, M_r 25000) that are distinct from the Ya monomer(s) (M_r 25500), but sample loading and polyacrylamide-gel performance are important factors if these are to be distinguished

electrophoretically. Under optimal conditions, Yk and Ya monomers can be resolved by SDS/polyacrylamide-gel electrophoresis; Yk monomer migrates faster towards the anode than does Ya monomer. Little difficulty was experienced in resolving Yf (M_r 24500) and Yk monomers. Moreover, these subunits are easily distinguished, since Yf possesses strong affinity for *S*-hexylglutathione-Sepharose 6B.

Both the amino acid compositions and immunochemical data (Hayes & Mantle, 1986b) suggest that transferase K may be structurally related to the group I enzymes (i.e. those that contain Ya and/or Yc subunits). Tryptic fingerprints support this conclusion, and show that the Yk subunit shares sequence homology with both Ya and Yc subunits but is more closely related to Ya than to Yc subunits.

The peptide mapping experiments suggest that the two subunits comprising transferase K are highly homologous. Indeed, it is probable that transferase K is a homodimer since tryptic digests of this enzyme yielded only 30 soluble peptides, though the dimeric protein contains about 63 arginine + lysine residues.

In conclusion, a new glutathione *S*-transferase has been isolated that exhibits novel chromatographic behaviour and unusual catalytic properties. This form is designated transferase K and comprises new subunits. The transferases represent a complex 'multi-gene' family. During the present study the structural relationship between transferase K and the Ya and Yc polypeptides in rat liver was investigated. The immunochemical relationship between Yk and other subunits is described in the accompanying paper (Hayes & Mantle, 1986b).

I thank the Medical Research Council for a project grant (G8126392 SB). I am greatly indebted to Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, for providing laboratory facilities, for performing the amino acid analyses and for his expert advice. I gratefully acknowledge the help of Mrs. M. Daniel and Mr. P. K. Stockman. I thank Professor L. G. Whitby for his continued generous support and Mrs. E. Ward for secretarial assistance.

REFERENCES

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
 Ambler, R. P. & Brown, L. H. (1967) *Biochem. J.* **104**, 784–825
 Beale, D., Ketterer, B., Carne, T., Meyer, D. J. & Taylor, J. B. (1982) *Eur. J. Biochem.* **126**, 459–463
 Beale, D., Meyer, D. J., Taylor, J. B. & Ketterer, B. (1983) *Eur. J. Biochem.* **137**, 125–129
 Boyer, T. D., Kenney, W. C. & Zakim, D. (1983) *Biochem. Pharmacol.* **32**, 1843–1850
 Chasseaud, L. F. (1979) *Adv. Cancer Res.* **29**, 175–274
 Frey, A. B., Freidberg, T., Oesch, F. & Kreibich, G. (1983) *J. Biol. Chem.* **258**, 11321–11325
 Gillham, B. (1973) *Biochem. J.* **135**, 797–804
 Habig, W. H. & Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 218–230
 Hayes, J. D. (1983) *Biochem. J.* **213**, 625–633
 Hayes, J. D. (1984) *Biochem. J.* **224**, 839–852
 Hayes, J. D. & Chalmers, J. (1983) *Biochem. J.* **215**, 581–588
 Hayes, J. D. & Clarkson, G. H. D. (1982) *Biochem. J.* **207**, 459–470
 Hayes, J. D. & Mantle, T. J. (1986a) *Biochem. J.* **233**, 407–415
 Hayes, J. D. & Mantle, T. J. (1986b) *Biochem. J.* **233**, 779–788
 Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1981) *Biochem. J.* **197**, 491–502
 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
 Lai, H.-C. J., Li, N.-Q., Weiss, M. J., Reddy, C. C. & Tu, C.-P. D. (1984) *J. Biol. Chem.* **259**, 5536–5542
 Mannervik, B. & Guthenberg, C. (1981) *Methods Enzymol.* **77**, 231–235
 Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J.-F., Argenbright, L. & Lu, A. Y. H. (1984) *J. Biol. Chem.* **259**, 5182–5188
 Simons, P. C. & Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
 Stockman, P. K., Beckett, G. J. & Hayes, J. D. (1985) *Biochem. J.* **227**, 457–465

Received 12 June 1985/28 August 1985; accepted 1 October 1985