Characterization of the basic glutathione *S*-transferase B₁ and B₂ subunits from human liver

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The basic glutathione *S*-transferases in human liver are composed of at least two immunochemically distinct polypeptides, designated B₁ and B₂. These subunits exist as homodimers, but can hybridize to form the B₁B₂ heterodimer [Stockman, Beckett & Hayes (1985) *Biochem. J.* **227**, 457–465]. Although these basic glutathione *S*-transferases possess similar catalytic properties, the B₂ subunit exhibits significantly greater selenium-independent glutathione peroxidase activity than subunit B₁. The use of the ligands haematin, tributyltin acetate and Bromosulphophthalein as inhibitors of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity clearly discriminate between the B₁ and B₂ subunits and should help facilitate their identification. Peptide mapping experiments showed that B₁ and B₂ are structurally distinct, but related, subunits; subunit B₁ yielded 43 tryptic peptides, seven of which were unique, whereas subunit B₂ yielded 40 tryptic peptides, four of which were unique.

INTRODUCTION

The cytosolic glutathione *S*-transferases (GSTs) are a complex supergene family of dimeric enzymes (Tu & Reddy, 1985; Hayes, 1986; Rothkopf *et al.*, 1986; Li *et al.*, 1986; Ding *et al.*, 1986) that provide the body with several protective mechanisms against toxic xenobiotics (Smith *et al.*, 1977; Chasseaud, 1979).

Like the cytosolic enzymes in the rat, the human GSTs have been divided into three classes, which have been designated basic, neutral and acidic (Awasthi *et al.*, 1980; Warholm *et al.*, 1983; Mannervik, 1985). The basic, neutral and acidic GST enzymes contain polypeptides that not only are immunochemically related to the rat Ya-, Yb- and Yf-type subunits but possess similar mobilities during SDS/polyacrylamide-gel electrophoresis (Hayes & Mantle, 1986*b,c*; Tu *et al.*, 1986).

Kamisaka *et al.* (1975) originally described a purification scheme for five basic GST forms (α , β , γ , δ and ϵ) from human liver, which differed in isoelectric point (pI 7.8–8.8) but were indistinguishable by other criteria. These workers proposed that GST α – ϵ were coded by a single gene and that the multiple forms were generated by a post-synthetic modification (Jakoby & Habig, 1980). We have since demonstrated that the basic human hepatic GST enzymes contain two immunochemically distinct subunits (Hayes *et al.*, 1983; Stockman *et al.*, 1985), designated B₁ and B₂; these have been shown to hybridize *in vitro*. The B₁B₁, B₁B₂ and B₂B₂ forms probably correspond to GST ϵ , δ and γ (Stockman *et al.*, 1985). Some uncertainty surrounds the original identity of GST α – ϵ (Stockman *et al.*, 1985), and as a result of the polymorphism associated with these enzymes in man it is possible that further basic GST subunits exist (Hussey *et al.*, 1986).

During the present study we have examined the catalytic and structural properties of GST B₁B₁, B₁B₂ and B₂B₂, to determine the significance of the immuno-

chemical differences reported for the B₁ and B₂ GST subunits. The data presented indicate that the inter-individual differences in the isoenzyme composition of basic GST will be reflected in inter-individual differences in GST activities.

MATERIALS AND METHODS

Chemicals

These were all obtained commercially. Haematin, Cibacron Blue, Bromosulphophthalein, lithocholic acid 3-sulphate and cholic acid were from Sigma Chemical Co., Poole, Dorset, U.K. Cholic acid was purified before use by recrystallization, three times, from ethanol. Triethyltin bromide, tributyltin acetate and triphenyltin chloride were purchased from ICN Biomedicals, K & K Laboratories Division, Plainview, NY, U.S.A. *S*-Hexylglutathione was synthesized by the method of Vince *et al.* (1971). The *S*-hexylglutathione affinity matrix was made by coupling the γ -glutamyl moiety of *S*-hexylglutathione to epoxy-activated Sepharose 6B (Mannervik & Guthenberg, 1981).

Analytical

The electrophoretic methods used have been described previously (Stockman *et al.*, 1985; Hayes & Mantle, 1986*c*).

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

Tryptic peptide mapping experiments were carried out on performic acid-oxidized protein by using methods devised by Ambler (1963) and Ambler & Brown (1967).

The antibodies used during the present study, and the immunization schedule by which they were obtained, have been described previously (Hayes *et al.*, 1983; Beckett & Hayes, 1984).

Abbreviation used: GST, glutathione *S*-transferase.

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Scheme 1. Purification of human GST enzymes

Purification of hepatic GST

Human livers were obtained within 8 h of death, at autopsy, from individuals (45–69 years old) who had died from disease processes that did not involve the liver.

The basic enzymes, B₁B₁, B₁B₂ and B₂B₂, and the neutral enzyme, GST μ, were isolated from human liver cytosol by using standard chromatographic procedures (Hayes *et al.*, 1983, 1987; Stockman *et al.*, 1985). A summary of the purification strategy used is shown in Scheme 1.

Purification of extrahepatic GST

An acidic class transferase, GST λ, was isolated from human lung by methods described elsewhere (Hayes *et al.*, 1987).

RESULTS

Polymorphism

The majority of the livers studied to date have been found to contain large amounts of GST B₁B₁ and B₁B₂. Relatively few livers were found to express significant amounts of GST B₂B₂; the liver from which this enzyme was isolated provided a yield of about 3 mg of B₂B₂ protein per 100 g of tissue. As already noted by Warholm *et al.* (1983) the neutral enzyme, GST μ, is found in only about 60% of liver specimens.

Electrophoretic properties of human GST

Portions of the basic, neutral and acidic GST enzymes were analysed by SDS/polyacrylamide-gel electrophoresis and isoelectric focusing. The basic subunits, B₁ and B₂, co-migrated during SDS/polyacrylamide-gel electrophoresis as single bands that ran between the Ya (*M_r* 25 500) and Yb (*M_r* 26 300) monomers from rat liver. Both B₁ and B₂ subunits have an apparent *M_r* of about 26 000. The subunits from GST μ migrated between the Yb (*M_r* 26 300) and Yc (*M_r* 27 500) monomers from the rat; the neutral subunits have an *M_r* of 26 700. The subunits from GST λ co-migrated with the rat Yf monomer; the acidic subunit has an *M_r* of about 24 800.

Isoelectric focusing of GST B₁B₁, B₁B₂ and B₂B₂ revealed that these enzymes had pI values of 8.9, 8.75 and 8.4 respectively, whereas GST μ and λ had pI values of 6.1 and 4.8 respectively. These data indicate that the GST enzymes obtained during the present study were homogeneous and closely similar to forms isolated previously in this laboratory from separate human liver specimens.

Substrate specificities of human GST

Table 1 lists the specific activity of each human liver enzyme for various GST substrates; also included for comparison are the values obtained for the acidic transferase from human lung, GST λ. All of the enzymes studied were highly active with 1-chloro-2,4-dinitro-

Table 1. Specific activities of human GST enzymes

N.D. denotes values that were not determined (because of shortage of material).

Substrate	Specific activity (μmol/min per mg at 37 °C)					
	GST ...	B ₁ B ₁	B ₁ B ₂	B ₂ B ₂	μ	λ
1-Chloro-2,4-dinitrobenzene		82	117	80	272	212
1,2-Dichloro-4-nitrobenzene		0.25	0.86	0.79	0	0.14
Ethacrynic acid		0.11	0.16	0.14	0.22	1.22
<i>trans</i> -4-Phenylbut-3-en-2-one		0	0	0	0.45	0.02
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane		0	0	0	N.D.	0.53
Cumene hydroperoxide		31	92	104	N.D.	0.11
<i>p</i> -Nitrophenyl acetate		0.66	0.97	0.24	N.D.	0.38

Table 2. Inhibition of human basic GST enzymes

The inhibition of GST activity was studied with 1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH as substrates at pH 6.5 and 37 °C. The I_{50} value is the concentration of inhibitor that decreased GST activity (under these conditions) to 50% (see, e.g., Hayes & Mantle, 1986a). Triethyltin bromide is listed first since it is the only compound tested that inhibited the B_2 subunit more than the B_1 subunit.

Inhibitor	GST . . .	I_{50} value (μ M)		
		B_1B_1	B_1B_2	B_2B_2
Triethyltin bromide		1.55	0.76	0.145
Tributyltin acetate		< 0.001	0.15	0.98
Triphenyltin chloride		0.3	0.7	1.5
Haematin		1.5	3	40
Cibacron Blue		2.5	7.8	24
<i>S</i> -Hexylglutathione		4.6	5.6	6.6
Bromosulphophthalein		10.5	20	125
Lithocholic acid 3-sulphate		6.6	28	36
Cholic acid		1.15×10^3	2.4×10^3	4.1×10^3

benzene, but some enzyme forms exhibited little or no detectable activity with other substrates. Of particular interest was the high glutathione peroxidase activity, with cumene hydroperoxide as substrate, of GST B_1B_1 , B_1B_2 and B_2B_2 . The B_1 and B_2 subunits appear to possess significantly different glutathione peroxidase II activities: the B_2 subunit exhibits a 3-fold greater activity for cumene hydroperoxide than does the B_1 subunit. The basic enzymes were less active with 1-chloro-2,4-dinitrobenzene but more active with 1,2-dichloro-4-nitrobenzene than were the other enzymes. By contrast, GST μ was characterized by a high specific activity with *trans*-4-phenylbut-3-en-2-one but was inactive with 1,2-dichloro-4-nitrobenzene. None of these enzymes displayed a high specific activity with ethacrynic acid, the property that is characteristic of GST λ .

It should be noted that the hybrid basic GST, B_1B_2 , possessed a significantly greater specific activity for substrates such as 1-chloro-2,4-dinitrobenzene and *p*-nitrophenyl acetate than did either of the homodimers, B_1B_1 and B_2B_2 .

Inhibition of basic GST activity

Table 2 shows the I_{50} results for the three basic GST enzymes with a variety of inhibitors. The organometal halides were the most potent of all the inhibitors, the greatest inhibition being produced by tributyltin acetate. GST B_1B_1 , B_1B_2 and B_2B_2 did not respond equally to the effects of each inhibitor. Small but significant differences (up to 6-fold) in the degree of inhibition of GST B_1B_1 compared with GST B_2B_2 were found with cholic acid, lithocholic acid 3-sulphate, *S*-hexylglutathione and triphenyltin chloride. However, tributyltin acetate was at least 1000 times more effective at inhibiting the activity of GST B_1B_1 than that of GST B_2B_2 . Haematin was about 30 times more effective with GST B_1B_1 than with GST B_2B_2 , and Cibacron Blue and Bromosulphophthalein also produced similar but less marked differences in the level of inhibition. Triethyltin bromide inhibited GST B_2B_2 about 10 times more than it did GST B_1B_1 , and was the only compound tested that was more effective with GST B_2B_2 than GST B_1B_1 .

All the I_{50} values obtained for the GST B_1B_2 heterodimer were found to lie between those of the

Table 3. Amino acid composition of human hepatic GST enzymes

Samples (0.5 mg of protein) were hydrolysed in 6 M-HCl for 24 h. The results were obtained in μ mol of amino acid recovered and from these values were calculated the percentage amino acid composition of each protein. The recoveries of cysteine and tryptophan were not determined and have been excluded from the calculations. The compositions were not corrected for the increased or decreased recoveries of serine, threonine, valine and isoleucine. Data for B_1B_1 GST and B_1B_2 GST were not significantly different from those for B_2B_2 GST and are not shown.

Amino acid	GST pI Subunit M_r . . .	Amino acid composition (%)	
		B_2B_2 8.4 26000	μ 6.1 26700
Lys		10.8	10.2
His		1.3	2.4
Arg		5.1	4.7
Asx		8.4	11.2
Thr		1.9	3.1
Ser		6.3	4.9
Glx		13.3	6.3
Pro		4.6	5.1
Gly		5.2	5.3
Ala		6.4	4.6
Val		3.9	3.4
Met		3.4	3.6
Ile		6.9	6.9
Leu		13.6	13.2
Tyr		4.4	6.0
Phe		4.4	6.4

corresponding homodimers, reflecting the catalytic contribution of the two functionally distinct subunits to the hybrid enzyme.

Amino acid compositions

All the basic enzymes studied were found to have closely similar amino acid compositions and could not be

Fig. 1. Map of the acidic tryptic peptides from basic GST enzymes

The tryptic digests of portions (1.5 mg of protein) of B_1B_1 , B_1B_2 and B_2B_2 GST enzymes were prepared. Peptides were separated by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 in the first dimension. The acidic peptides were cut out of the paper and separated in the second dimension by high-voltage paper electrophoresis at pH 3.5. The stained peptide spots were traced to produce the diagram. A mixture of amino acids (Ambler, 1963) was run in parallel to facilitate comparisons between the maps; the abbreviations employed to indicate the mobilities of these markers have been described elsewhere (Hayes, 1986). Peptides common to all three GST enzymes are represented by blackened spots. The peptide that appeared unique to the B_1 subunit is not shaded. The peptide that appeared unique to the B_2 subunit is shown by the hatched spot.

discriminated on this basis. However, the recoveries of tyrosine, phenylalanine, aspartic acid and glutamic acid obtained from the neutral enzyme, GST μ , were significantly different from the values obtained from the basic GST forms (Table 3).

Peptide mapping of basic GST enzymes

Diagrammatic representations of the acidic, neutral and basic tryptic peptides obtained from oxidized portions of B_1B_1 , B_1B_2 and B_2B_2 proteins are shown in Figs. 1–3.

Examination of the maps of the acid peptides (Fig. 1) revealed that seven were common to GSH B_1B_1 , B_1B_2 and B_2B_2 . One acidic peptide (no shading) was found only in the B_1 -containing GST enzymes, B_1B_1 and B_1B_2 . A further acidic peptide (hatched shading) was only recovered from the B_2 -containing GST enzymes, B_2B_2 and B_1B_2 .

The neutral peptide maps (Fig. 2) demonstrated the existence of ten peptides shared by GST B_1B_1 , B_1B_2 and B_2B_2 . Four additional tryptic peptides were observed in GST B_1B_1 and B_1B_2 (no shading), but not in GST B_2B_2 . Conversely, three peptides were found in GST B_1B_2 and B_2B_2 (hatched shading), but not in GST B_1B_1 .

The basic peptide maps (Fig. 3) revealed 19 tryptic peptides common to GST B_1B_1 , B_1B_2 and B_2B_2 . Two additional peptides appear to be specific for the B_1

subunit (no shading); no basic peptides were recovered that were specific for the B_2 subunit.

The tryptic peptides that appeared to be specific for the B_1 and B_2 subunits, the 'difference peptides', occurred in a manner consistent with the hypothesis that GST B_1B_2 is a heterodimer comprising a subunit common to GST B_1B_1 and a subunit common to GST B_2B_2 . No peptides were found to be unique to any one of the basic GST enzymes.

Immunoblotting

The polyclonal antisera previously employed in radioimmunoassays to discriminate between B_1 and B_2 polypeptides (Beckett & Hayes, 1984; Stockman *et al.*, 1985) were used to probe these subunits following transfer to nitrocellulose. Neither anti- $(B_1$ subunit) nor anti- $(B_2$ subunit) serum could distinguish between the two basic subunits in Western-blotting experiments, despite their 300–500-fold difference in cross-reactivity in the radioimmunoassays. This method-dependent difference in immunoreactivity reflects the fact that the radioimmunoassay technique is used to challenge GST in their native, active, state, whereas the Western-blot technique probes denatured GST subunits in random-coil configuration. These data are consistent with the close sequence homology between subunits B_1 and B_2 that is evident from the peptide 'mapping' experiments.

Fig. 2. Map of the neutral tryptic peptides from basic GST enzymes

The neutral tryptic peptides from portions (1.5 mg of protein) of B_1B_1 , B_1B_2 and B_2B_2 GST enzymes were isolated by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5. These were resolved in the first dimension by high-voltage paper electrophoresis at pH 3.5. Separation in the second dimension was performed by descending chromatography using butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h. The stained peptide spots were traced to produce the diagram. Peptides common to all 'basic' GST enzymes are represented by blackened spots. The four neutral peptides that appeared unique to the B_1 subunit are not shaded, and the three neutral peptides unique to the B_2 subunit are shown by the hatched spots.

Fig. 3. Map of the basic tryptic peptides from basic GST enzymes

The map of the basic tryptic peptides was constructed by high-voltage paper electrophoresis (h.v.p.e.) at pH 6.5 in the first dimension, and descending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) in the second dimension. The ninhydrin-positive peptides were traced to produce the diagram. The blacked spots represent common peptides, and the spots that are not shaded represent peptides unique to the B_1 subunit.

DISCUSSION

The cytosolic GST enzymes in man have been divided into three groups, which are thought to represent the products of different gene families; these have been referred to, according to their pI values, as the basic, the neutral and the acidic GST. These genetic relationships have been concluded from data (Warholm *et al.*, 1983; Koskelo, 1983; Dao *et al.*, 1984; Ålin *et al.*, 1985; Stockman *et al.*, 1985; Vander Jagt *et al.*, 1985; Soma *et al.*, 1986) that indicated the three classes of human GST comprise structurally and immunochemically distinct subunits that are functionally separate.

Relatively little is known about the relationships between individual enzymes within the three classes of human GST, but, to date, the basic group of GST enzymes has been most extensively studied. Previous work from this laboratory described two antisera that, in radioimmunoassays, could discriminate between members of this group of GST (Beckett & Hayes, 1984). This finding led us to suggest the presence of two separate basic GST polypeptides, B₁ and B₂, in human liver that could hybridize and were found as B₁B₁, B₁B₂ and B₂B₂ subunit combinations (Stockman *et al.*, 1985). The fact that the specific anti-(B₁ subunit) and anti-(B₂ subunit) sera are polyclonal suggests it is improbable that a post-synthetic modification could account for the differences in the B₁ and B₂ subunits.

The peptide 'mapping' experiments described in Figs. 1–3 not only support the hypothesis that a hybrid basic GST exists in man, but also show that the B₁ and B₂ subunits are structurally distinct. These data suggest that the B₁ and B₂ subunits are genetically separate but share a significant sequence homology (possibly > 90% identity). With this degree of homology it is likely that the 'difference peptides' are exposed on the surface of the protein, since the immunochemical differences that exist between the B₁ and B₂ subunits are only observed by using radioimmunoassay but are not seen with Western blotting.

During the present study catalytic differences between the B₁ and B₂ subunits have been established; the B₂ subunit has been found to exhibit a significantly greater selenium-independent glutathione peroxidase activity than that of the B₁ subunit. Large differences have also been revealed in the susceptibilities of the B₁ and B₂ subunits to inhibition by tributyltin acetate, triethyltin bromide, Bromosulphophthalein and haematin. These differences in the functional properties of the B₁ and B₂ subunits may suggest that at least one of the 'difference peptides' described above reside near the hydrophobic binding domain.

Inhibitors of GST activity have been used to discriminate between related subunits in the rat (Hayes & Mantle, 1986a; Tahir & Mannervik, 1986). The inhibitors used in the present study provide a simple means of distinguishing between the two human GST subunits B₁ and B₂. Of all the compounds tested the majority were found to inhibit subunit B₁ more than subunit B₂; tributyltin and haematin showed the largest difference in inhibition of subunit B₁ relative to subunit B₂. By contrast, triethyltin bromide showed a 10-fold greater inhibition of subunit B₂ than of subunit B₁.

Polymorphism of the neutral Yb-containing GST in man has been recognized (Warholm *et al.*, 1980; Board, 1981; Strange *et al.*, 1984; Hussey *et al.*, 1986). Marked

variations also exist in the content of basic GST in liver specimens. The differences in the catalytic and ligand-binding activities of the B₁ and B₂ subunits indicate that the basic GST may be responsible for some of the variations within the population in drug metabolism and in susceptibility to cytotoxic compounds.

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