Evidence that glutathione *S*-transferases B₁B₁ and B₂B₂ are the products of separate genes and that their expression in human liver is subject to inter-individual variation

Molecular relationships between the B₁ and B₂ subunits and other Alpha class glutathione *S*-transferases

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The Alpha class glutathione *S*-transferases (GSTs) in human liver are composed of polypeptides of *M_r* 25900. These enzymes are dimeric, and two immunochemically distinct subunits, B₁ and B₂, have been described that combine to form GSTs B₁B₁, B₁B₂ and B₂B₂ [Stockman, Beckett & Hayes (1985) *Biochem. J.* **227**, 457–465]. Gradient affinity elution from GSH–Sephacrose has been used to resolve the three Alpha class GSTs, and this method has been applied to demonstrate marked inter-individual differences in the hepatic content of GSTs B₁B₁, B₁B₂ and B₂B₂. The B₁ and B₂ subunits can be resolved by reverse-phase h.p.l.c., and their elution positions suggest that they are equivalent to the α_x and α_y h.p.l.c. peaks described by Ketterer and his colleagues [Ostlund Farrants, Meyer, Coles, Southan, Aitken, Johnson & Ketterer (1987) *Biochem. J.* **245**, 423–428]. The B₁ and B₂ subunits have now been cleaved with CNBr and the fragments subjected to automated amino acid sequence analysis. The sequence data show that B₁ and B₂ subunits do not arise from post-translational modification, as had been previously believed for the hepatic Alpha class GSTs, but are instead the products of separate genes; B₁ and B₂ subunits were found to contain different amino acid residues at positions 88, 110, 111, 112, 116, 124 and 127. The relationship between the B₁ and B₂ subunits and the cloned *GTH₁* and *GTH₂* cDNA sequences [Rhoads, Zarlengo & Tu (1987) *Biochem. Biophys. Res. Commun.* **145**, 474–481] is discussed.

INTRODUCTION

The glutathione *S*-transferases (GSTs) are a complex group of detoxication enzymes that catalyse the conjugation of GSH with a large number of xenobiotics (Chasseaud, 1979; Ketterer *et al.*, 1988). Throughout Nature these enzymes play an important role in protecting cells against alkylating agents, and it is becoming apparent that, through over-expression or population polymorphisms, GST can confer drug-resistance to organisms (Hayes & Wolf, 1988).

Numerous GSTs exist, each with different catalytic and physicochemical properties. In man, four classes of GST have been identified that appear to be encoded by separate families of genes (Board, 1981; Strange *et al.*, 1984; Hayes, 1989). Three of these are cytosolic and are called Alpha, Mu and Pi class GSTs (Mannervik *et al.*, 1985), and the remaining enzyme is membrane-bound and is called microsomal GST (De Jong *et al.*, 1988; McLellan *et al.*, 1989); the human Alpha, Mu and Pi class GSTs used to be referred to as the basic, neutral and acidic type enzymes respectively. In man, the Alpha, Mu and Pi class GSTs comprise subunits of *M_r* 25900, 26600–26700 and 24800 respectively, whereas the micro-

somal GST is composed of subunits of *M_r* 17300 (Hayes, 1989; McLellan *et al.*, 1989).

Besides their ability to form thioether linkages between GSH and toxic electrophiles, the GSTs possess additional activities. In particular, the multiple functions of the Alpha class enzymes have been recognized for many years. This class exhibits selenium-independent glutathione peroxidase activity (Awasthi *et al.*, 1980) as well as oxosteroid isomerase activity (Benson *et al.*, 1977). Moreover, Alpha class GSTs are sometimes referred to as 'ligandin' because they bind non-covalently non-substrate ligands, such as bilirubin, bile acids and penicillin, and bind covalently a number of carcinogens such as 4-aminoazobenzene and 3-methylcholanthrene (Litwack *et al.*, 1971; Kirsch *et al.*, 1975; Ketterer *et al.*, 1976; Hayes *et al.*, 1979).

Kamisaka *et al.* (1975) first described a purification scheme for several Alpha class GSTs from human liver, and at that time it was thought that the multiple forms that were isolated and called α , β , γ , δ and ϵ were generated by a post-synthetic modification, possibly deamidation (Jakoby & Habig, 1980). Subsequently, it has been shown that the hepatic Alpha class enzymes comprise two immunochemically distinct subunits, desig-

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

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nated B₁ and B₂, that combine to form the three dimeric enzymes, GST B₁B₁, GST B₁B₂ and GST B₂B₂ (Stockman *et al.*, 1985). These workers showed that GSTs B₁B₁, B₁B₂ and B₂B₂ represent respectively the ϵ , δ and γ forms of GST described by Kamisaka *et al.* (1975). The B₁ and B₂ subunits are closely homologous, but because they yield different peptide maps Stockman *et al.* (1987) proposed that they are the products of separate genes. However, it should be noted that hitherto no amino acid sequence data have been presented to support the hypothesis that B₁ and B₂ subunits are genetically distinct and that to date this hypothesis has been unproven.

At a nucleic acid level, Tu and his colleagues (Tu & Qian, 1986; Rhoads *et al.*, 1987) have described two separate full-length cDNA clones, *GTH*₁ and *GTH*₂, which code for human Alpha class GSTs. The subunits that are encoded by these cDNA clones are called Ha₁ and Ha₂; although these polypeptides share 96% sequence identity, no attempt has been made to determine what their exact relationship is with the GST B₁B₁ and GST B₂B₂ enzymes. At a protein level, Ostlund Farrants *et al.* (1987) have used reverse-phase h.p.l.c. to resolve the Alpha class GSTs into two peaks, designated α_x and α_y ; a formal comparison between α_x and α_y has not yet been described. The relationship between the α_x and α_y h.p.l.c. peaks and either B₁ and B₂ subunits or Ha₁ and Ha₂ subunits is unclear, and an appropriate study has not been undertaken. Clearly, in order to rationalize the literature it is necessary to determine the molecular relationship between B₁ and B₂ subunits, and establish how they compare with the Ha₁ and Ha₂ subunits and the α_x and α_y h.p.l.c. peaks.

Although B₁ and B₂ subunits can be distinguished by immunochemical methods, antibodies that can discriminate between these two subunits are not widely available. The identification of the B₁B₁ and B₂B₂ homodimers is not simple because they have many features in common and, unless both GST B₁B₁ and GST B₂B₂ are available, other criteria (such as pI value and enzyme activities) that help distinguish between members of the Alpha class GSTs cannot be applied with any certainty. GSTs in man are subject to population polymorphisms, and the marked variation in the GST content of different liver specimens also complicates the identification of the peaks obtained from chromatography columns. To compound the problem of identification, GST B₂B₂ adsorbs strongly to anion-exchangers and is eluted at an anomalous and variable position from the chromatofocusing matrix PBE 94; this chromatographic behaviour led Hayes *et al.* (1983) to refer originally to GST B₂B₂ as N/A2b because it was eluted in the region of the chromatogram occupied by neutral and acidic proteins.

During the present study we have established several methods that can be used to distinguish between the B₁ and B₂ subunits. These methods will allow researchers who do not have access to monospecific antisera to identify GSTs B₁B₁, B₁B₂ and B₂B₂. Gradient affinity elution from columns of GSH-Sepharose has been used to resolve these three hepatic Alpha class enzymes and is recommended as an aid to identification. The technique described is valuable because it can be used as a screening procedure to identify liver specimens that lack GST B₁B₁ or GST B₂B₂. The B₁ and B₂ subunits can be resolved and identified by using essentially the same reverse-phase h.p.l.c. method described by Ostlund Farrants *et al.* (1987). Sequence analysis of peptides obtained from

CNBr digests of B₁ and B₂ subunits has been undertaken and has provided the first definitive proof that B₁ and B₂ subunits are the products of separate genes. The relationship between B₁ and B₂ subunits, α_x and α_y h.p.l.c. peaks and Ha₁ and Ha₂ subunits is discussed.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the highest purity that was commercially available. The h.p.l.c. solvents were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland.

Buffers

These were all prepared at 20 °C, but the pH values quoted are those obtained at the temperature at which they were used (i.e. 4 °C).

Tissue

The livers used as enzyme source are specimens 5, 6 and 8 described by Hussey *et al.* (1986). Liver 5 was from a 42-year-old male, whereas both livers 6 and 8 were from 34-year-old males. All three individuals had died from accidental causes and the liver specimens, which were obtained within 2 h of death, were stored at -80 °C until required.

Enzyme purification

Portions of liver (100–250 g) were allowed to thaw at room temperature before being blended at 4 °C in 3 vol. of 50 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl (buffer A). The 100 000 g supernatants were prepared and passed through a plug of glass-wool before being dialysed for 18 h against three changes, each of 2 litres, of buffer A containing 0.5 mM-dithiothreitol. The dialysed samples were re-centrifuged at 20 000 g for 30 min, to remove denatured protein, before the supernatants were applied to 1.6 cm × 40.0 cm columns of GSH-Sepharose. These affinity columns were equilibrated with buffer A and, after the application of human liver cytosol, they were washed with about 600 ml of buffer A before the GST enzymes were eluted. Gradient elution, which resolved several peaks of activity, was performed with two separate gradients of counter-ligand formed from solutions (4 °C) of 2.5 mM-GSH and 50.0 mM-GSH in 200 mM-Tris/HCl buffer, pH 9.0 (buffer B), that were prepared immediately before use.

The individual peaks of GST activity that were eluted from the affinity matrix were subjected to DEAE-Sephadex chromatography, since this was the method that had been used originally by Hayes *et al.* (1983) to resolve GSTs B₁B₁, B₁B₂ and B₂B₂; in that study GSTs B₁B₁, B₁B₂ and B₂B₂ were called basic, N/A1 and N/A2b respectively. For this purpose the combined fractions from GSH-Sepharose were dialysed against four changes, each of 2 litres, of 20 mM-Tris/HCl buffer, pH 7.8 (buffer C), before being applied to 1.6 cm × 40 cm columns of DEAE-Sephadex; these contained a 1.6 cm × 5.0 cm plug of Sephadex on which the DEAE-Sephadex was overlaid. The column was equilibrated with buffer C and developed with 400 ml linear 0–175 mM-NaCl gradients that were formed in the same running buffer.

Enzyme assays

These were performed at 37 °C by the method of Habig & Jakoby (1981) with the use of a centrifugal fast analyser.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970) with a 12 % (w/v) polyacrylamide resolving gel that contained 0.32 % (w/v) *NN'*-methylenebisacrylamide [see Hayes & Mantle (1986) for details of the effects of cross-linker on the electrophoretic behaviour of Alpha class GSTs].

Reverse-phase h.p.l.c.

The 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 μ Bondapak C₁₈ column (10 μ m particle size; 0.39 cm $\times$ 30.0 cm) was from Millipore, Harrow, Middx., U.K. All samples for h.p.l.c. were centrifuged (10000 *g* for 5 min) in a micro-centrifuge to remove particulate material. For all applications the gradient controller was programmed to include a 5 min 'loading time' during which the sample was applied isocratically (i.e. 100 % solvent A) and the flow rate was increased linearly from 0.1 to 1.0 ml/min. The B₁ and B₂ subunits were resolved by using linear 40–58 % (v/v) gradients of acetonitrile in aq. 0.1 % (v/v) trifluoroacetic acid; these conditions are similar to those used by Ostlund Farrants *et al.* (1987) to resolve the α_x and α_y peaks.

CNBr cleavage

Portions (4 mg) of GSTs B₁B₁ and B₂B₂ were reduced and dialysed extensively against 20 mM-ammonium acetate buffer, pH 7.4. The dialysed proteins were freeze-dried and dissolved in 4.0 ml of aq. 70 % (v/v) formic acid before a crystal of approx. 25 mg of CNBr was added to each vial. Digestion was left to proceed in the dark for 20 h at room temperature before the reaction mixtures were diluted with 20 vol. of water and freeze-dried. The freeze-dried cleaved proteins were re-dissolved in 4.0 ml of aq. 0.1 % (v/v) trifluoroacetic acid, and the peptides were resolved by reverse-phase h.p.l.c. as described above, but with 0–70 % (v/v) acetonitrile gradients in aq. 0.1 % (v/v) trifluoroacetic acid to develop the μ Bondapak C₁₈ column.

Amino acid sequencing

Fractions from the μ Bondapak C₁₈ h.p.l.c. column were checked for purity before being sequenced by using an Applied Biosystems 130A Microbore Separation System (Applied Biosystems, Warrington, Cheshire, U.K.). The column employed was an Aquapore RP-300 (7 μ m particle size; 2.1 mm $\times$ 30 mm), also from Applied Biosystems, that was developed with a linear 8–80 % (v/v) gradient of acetonitrile in aq. 0.1 % (v/v) trifluoroacetic acid and monitored at 220 nm.

The pure peptides were subjected to automated sequencing on an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser. Polybrene (2 mg) was loaded on to a glass-fibre filter disc, which was then pre-cycled three times before being loaded with approx. 1.5 nmol of the CNBr-cleavage fragment.

After Edman degradation the anilinothiazolinone derivatives cleaved from the peptide were converted automatically into the more stable phenylthiohydantoin forms and separated on an Applied Biosystems PTH C₁₈ (5 μ m particle size; 2.1 mm $\times$ 220 mm) column that was developed with a 0–100 % (v/v) linear gradient of acetonitrile. The gradient was formed by using an aq. 5 % (v/v) solution of tetrahydrofuran as solvent A and acetonitrile as solvent B. Chromatography was performed at 55 °C and the column was monitored at 269 nm.

Vapour-phase pyridylethylation of cysteine residues

The CNBr-cleavage fragment requiring pyridylethylation (B₁-CNBr7) was spotted on to a glass-fibre filter disc and dried under a stream of N₂. The pyridylethylation reaction was done in a stoppered glass tube (50 mm $\times$ 10 mm) with a constriction in the middle to support the filter. Pyridine (100 μ l), water (100 μ l), 4-vinylpyridine (20 μ l) and tributylphosphine (20 μ l) were placed in the lower part of the tube. The tube was flushed thoroughly with Ar and the peptide-coated disc was placed on the constriction before the tube was sealed quickly with a stopper. The vapour-phase reaction was allowed to continue for 2 h at 60 °C (problems with condensation at the top of the tube can be minimized by warming the entire tube). Subsequently, the disc was removed from the tube, avoiding touching any liquid, and washed for 10 s in 2 ml of each of the three following solvents: *n*-heptane, *n*-heptane/ethyl acetate (1:1, v/v) and ethyl acetate. The washed disc was allowed to dry at room temperature before being wetted with 30 μ l (2 mg) of Polybrene. The Polybrene-treated disc was re-dried and placed in the cartridge block of the 477A sequencer. To remove all traces of reagent, the sample was pre-cycled with a wash programme (*n*-heptane followed by butyl chloride, 12.5 % trimethylamine in water, *n*-heptane and ethyl acetate) before the normal sequencing programme was begun. If the disc is contaminated with pyridine it is manifested as a broad peak in the middle of the chromatogram from the PTH C₁₈ column. The pyridylethylcysteine phenylthiohydantoin derivative is resolved on the PTH C₁₈ column with the standard separation protocol used by the on-line model 120 phenylthiohydantoin analyser and is eluted just before diphenylthiourea.

RESULTS

Resolution of GST enzymes on the GSH-Sephrose affinity matrix

Gradient affinity elution from GSH-Sephrose was used to prepare the human hepatic GSTs. Optimal resolution was obtained by employing two separate 400 ml gradients of GSH in buffer B to develop the matrix. An initial, shallow, 0–2.5 mM gradient of GSH was found to elute two major peaks of GST activity; these were combined separately and are referred to as pools 1 and 2 (Fig. 1). A second, steeper, 2.5–50.0 mM gradient of GSH eluted a third major peak of GST activity, which was collected and is called pool 3. Pool 1 (fractions 28–38), pool 2 (fractions 65–85) and pool 3 (fractions 108–131) were eluted at GSH concentrations of about 0.50, 2.00 and 21.0 mM respectively. GSTs were prepared from three separate livers by using this method

Fig. 1. Gradient elution of human GSTs from GSH-Sepharose

Hepatic cytosols from three human livers (specimens 5, 6 and 8) were prepared separately and dialysed as described in the text. After re-centrifugation, portions of the cytosol from these three specimens (liver 5, 19 g of protein; liver 6, 11.1 g of protein; liver 8, 21.5 g of protein) were applied to 1.6 cm $\times$ 40.0 cm columns of GSH-Sepharose. The affinity matrix was washed (32 ml/h) with 600–800 ml of buffer A before the GST enzymes were eluted. Columns were developed in two stages with two separate continuous gradients of GSH in buffer B (i.e. 0–2.5 mM over 400 ml, followed by 2.5–50 mM also over 400 ml). Fractions (5.0 ml) were collected and GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) ($\blacktriangle$) and A_{280} ($\circ$) were measured. The GSH gradient is represented by a continuous line. In each panel the liver specimen examined is indicated. The fractions that were combined and designated pools 1–3 are indicated by the horizontal bars.

Fig. 2. Reverse-phase h.p.l.c. of human GSTs and its use to resolve B₁ and B₂ subunits

Portions of purified GST B₁B₂ were subjected to h.p.l.c. on a Waters μ Bondapak C₁₈ column (10 μ m particle size; 0.39 cm $\times$ 30.0 cm). The column was developed at 1 ml/min by a linear 40–58% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid formed over 60 min that was followed by a 58–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous straight lines; pump A delivered 40% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid and pump B delivered 70% (v/v) acetonitrile also in aq. 0.1% (v/v) trifluoroacetic acid.

and each provided a distinct elution profile. Liver 5 contained pools 2 and 3, liver 6 contained pools 2 and 3 and liver 8 contained primarily pool 1. Examination of the peak from liver 6 that was eluted between 10 mM- and 28 mM-GSH (i.e. pool 3) showed it to be clearly asymmetric, suggesting the presence of more than one component. This asymmetry was not present in the profile from liver 5.

SDS/polyacrylamide-gel electrophoresis was performed to establish the subunit types present in the various pools from the three livers. In all cases pools 1, 2 and 3 contained a polypeptide(s) of M_r 25900 that comigrated with the Alpha class B₁ and B₂ subunits. However, in the early fractions of pool 3 from liver 6 an additional polypeptide was observed with a lower mobility and an estimated M_r of 26700; this subunit, which was not detected in either liver 5 or liver 8, comigrated with the Mu class Yb subunits. Besides these subunits a number of polypeptides with an M_r greater than 35000 were eluted from the GSH–Sephrose column immediately before pool 1 (i.e. in fractions 20–24). This material, which was eluted at very low concentrations of GSH (0.25 mM), was detected in all three livers, but had little, if any, activity towards 1-chloro-2,4-dinitrobenzene.

Fig. 3. Analytical and preparative peptide maps of B₁ and B₂ subunits

CNBr digests of portions (4 mg) of both GST B₁B₁ and GST B₂B₂ were prepared simultaneously, as described in the text. The resulting CNBr-cleavage fragments were dissolved in 4 ml of aq. 0.1% (v/v) trifluoroacetic acid and, after centrifugation, portions (1 mg of protein) were applied to a Waters μ Bondapak C₁₈ column (10 μ m particle size; 0.39 cm $\times$ 30 cm) that had been equilibrated with aq. 0.1% (v/v) trifluoroacetic acid. The μ Bondapak column was developed with a 0–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid that was formed over 75 min. The continuous straight line represents the output of pump B; pump A delivered aq. 0.1% (v/v) trifluoroacetic acid and pump B delivered 70% (v/v) acetonitrile also in aq. 0.1% (v/v) trifluoroacetic acid. Panels (a) and (b) show the chromatograms obtained with GST B₁B₁ and GST B₂B₂ respectively.

Fig. 4. Primary structures of subunits B₁ and B₂

The sequences of CNBr-derived peptides from subunits B₁ and B₂ are shown aligned for comparison with the homologous sequences deduced from the cDNA *GTH*₁ (Tu & Qian, 1986) and cDNA *GTH*₂ (Rhoads *et al.*, 1987) respectively. Putative methionine residues are in parentheses. The residues marked by an asterisk (*) are those that differ between the proteins that would be encoded by *GTH*₁ and *GTH*₂ cDNAs (Rhoads *et al.*, 1987). Residue 111 is underlined because of the divergence between the amino acid residue found in subunit B₂ and the amino acid that was predicted from *GTH*₂ cDNA. It should be noted that as peptide CNBr-7 has been sequenced through 50 cycles it occupies two lines. Further, the sequence of peptide CNBr-2 was obtained for the B₁ subunit but not the B₂ subunit.

Identification of B₁-containing and B₂-containing GSTs

As pools 1, 2 and 3 all contained Alpha class subunits of *M_r* 25900, each was subjected to DEAE-Sephadex

chromatography to allow the GSTs that they contained to be identified. Pool 1 failed to bind to DEAE-Sephadex and, as it was eluted in the flow-through fractions, it was identified as GST B₁B₁. Pool 2 was retained by the anion-

exchanger and, being eluted by the salt gradient at an Na⁺ concentration of about 54 mM (result not shown), it was identified as GST B₁B₂. Examination of pool 3 by DEAE-Sephadex chromatography gave different profiles for liver specimens 5 and 6, as would be predicted from the SDS/polyacrylamide-gel electrophoresis results described above. In both cases the major peak of activity bound to the anion-exchanger strongly and was eluted by the salt gradient at an Na⁺ concentration of 98 mM. This peak contained subunits of *M_r* 25900 (result not shown) and therefore represented GST B₂B₂.

Portions of the peaks from DEAE-Sephadex that were identified as GSTs B₁B₁, B₁B₂ and B₂B₂ were withdrawn and their immunoreactivities with anti-(GST B₁B₁) IgG and anti-(GST B₂B₂) IgG were determined. The selection of antibodies and the radioimmunoassay procedure is described elsewhere (Beckett & Hayes, 1984). The results from these radioimmunoassay experiments showed that the DEAE-Sephadex-chromatography peak from pool 1, identified as GST B₁B₁, had less than 5% cross-reactivity with anti-(GST B₂B₂) IgG when compared with the immunogen. However, this peak showed the same cross-reactivity as GST B₁B₁ (i.e. 100%) in the anti-(B₁ subunit) radioimmunoassay. Similarly, the DEAE-Sephadex peak from pool 3, identified as GST B₂B₂, had less than 5% cross-reactivity with anti-(GST B₁B₁) IgG when compared with the immunogen but displayed the same cross-reactivity as GST B₂B₂ in the anti-(B₂ subunit) radioimmunoassay. The peak that was identified as GST B₁B₂ gave the same reactivity signal in both the anti-(B₁ subunit) and the anti-(B₂ subunit) radioimmunoassays. Hence the immunochemical data are entirely consistent with the identifications of the Alpha class GSTs that were made on the basis of chromatographic behaviour.

Identification of B₁ and B₂ subunits as α_x and α_y h.p.l.c. peaks

Ostlund Farrants *et al.* (1987) have described the resolution of Alpha class GSTs from human liver into two peaks, α_x and α_y , by reverse-phase h.p.l.c. These workers defined α_x as the first Alpha class GST to be eluted from the reverse-phase column whereas α_y was designated as the second peak to be eluted. To help establish which of the purified human hepatic GSTs represent the α_x and α_y h.p.l.c. peaks, portions of purified GST B₁B₁, GST B₁B₂ and GST B₂B₂ were subjected to reverse-phase h.p.l.c. as described in the Materials and methods section. Under these conditions, which included the 5 min 'loading time,' the subunits from GST B₁B₁ and GST B₂B₂ were both eluted from the reverse-phase column as single peaks at 46 min and 50 min respectively. Fig. 2 shows the elution of GST B₁B₂ from the μ Bondapak column. It is concluded from these data that the α_x and α_y h.p.l.c. peaks represent the B₁ and B₂ subunits respectively.

Evidence that B₁ and B₂ subunits are genetically distinct and evaluation of their relationship with *GTH*₁ and *GTH*₂ cDNA clones

Protein sequencing experiments were undertaken for two reasons. Firstly, sequence data were required to allow the genetic origins of B₁ and B₂ subunits to be established. Secondly, information about the primary structure of B₁ and B₂ subunits would allow comparison with the protein structures deduced from DNA sequence analysis of the *GTH*₁ and *GTH*₂ clones.

The DNA sequence data reported by Tu and his colleagues (Rhoads *et al.*, 1987) suggest the presence of seven methionine residues (excluding the initiation codon) at positions 15, 50, 56, 62, 93, 104 and 207 in both *GTH*₁ and *GTH*₂ clones; hence the proteins that these cDNAs encode would be expected to give rise to eight CNBr-cleavage fragments. Purified portions of GSTs B₁B₁ and B₂B₂ were subjected to CNBr cleavage and the resulting peptides were resolved by reverse-phase h.p.l.c. on the μ Bondapak column. With the use of the h.p.l.c. as both an analytical and a preparative tool it can be seen from Fig. 3 that CNBr digests of GSTs B₁B₁ and B₂B₂ gave rise to closely similar profiles. All the *A*₂₂₀ peaks from GST B₁B₁ were collected and, if pure, were subjected to a minimum of five automated Edman cycles to identify to which of the eight predicted CNBr-cleavage peptides they corresponded.

A comparison of the protein sequencing data with the DNA sequencing data allowed five of the eight putative CNBr-cleavage fragments to be identified; the material that was eluted from the μ Bondapak column at 56 min corresponded to the expected peptide CNBr-2 (residues 16–50), that at 49 min to peptide CNBr-5 (residues 63–93), that at 41.5 min to peptide CNBr-6 (residues 94–104), that at 62.5 min to peptide CNBr-7 (residues 105–207) and that at 44.5 min to peptide CNBr-8 (residues 208–221). The peptide-containing peaks in Fig. 3(a) and 3(b) that are designated a, b and c represent the CNBr-8, CNBr-5 and CNBr-7 fragments respectively. These peaks are of particular significance because they represent the 'difference peptides' (see below).

The peptides CNBr-2, CNBr-5, CNBr-6, CNBr-7 and CNBr-8 from GST B₁B₁ were subjected to 20, 30, 10, 50 and five automated Edman degradation cycles respectively (Fig. 4). For comparative purposes the peptides CNBr-5, CNBr-6, CNBr-7 and CNBr-8 from GST B₂B₂ were also subjected to sequence analysis (Fig. 4). Over the common regions sequenced (residues 62–154) B₁ and B₂ subunit contains lysine. At residues 110–112 subunit B₁ differences, which proves that they are genetically separate. At residue 88 B₁ subunit contains arginine whereas B₂ subunit contains lysine. At residues 110–112 subunit B₁ possesses Val-Cys-Pro whereas subunit B₂ contains Phe-Ser-Gln; the cysteine residue was identified by a separate analytical run after pyridylethylation of peptide CNBr-7 (see the Materials and methods section for details). At positions 116, 124 and 127 subunit B₁ possesses lysine, lysine and isoleucine, but by contrast at these positions subunit B₂ has glutamine, glutamine and threonine. The sequence analysis revealed that subunit B₁ corresponds exactly to the *GTH*₁ cDNA (Fig. 4). These data also revealed that subunit B₂ corresponds almost exactly to the *GTH*₂ clone; Fig. 4 shows a single difference at position 111, where subunit B₂ contains serine rather than threonine, which is the amino acid encoded for by *GTH*₂ cDNA.

DISCUSSION

Previous work has shown that the Alpha class GST is represented in human liver by multiple enzymes. Originally, Kamisaka *et al.* (1975) isolated five forms, designated α , β , γ , δ and ϵ , which were considered at the time to be charge isomers produced by deamidation *in vivo*. It has also been proposed that proteolysis or autoxidation are responsible for the production of the multiple forms

[for further details of the possible influence of these mechanisms see Grover (1982) and Suzuki *et al.* (1987)]. However, other workers have provided data that cast doubt on the hypothesis that the multiple enzymes α - ϵ arise as a consequence of post-translational modification (Hayes *et al.*, 1983; Stockman *et al.*, 1985, 1987; Soma *et al.*, 1986; Sugimoto *et al.*, 1987). These workers have reported that the Alpha class enzymes in human liver comprise two subunits that are immunochemically and catalytically distinct. These two subunits have been called B₁ and B₂ by Stockman *et al.* (1985), or Y₁ and Y₄ by Sato and his colleagues (Soma *et al.*, 1986; Tateoka *et al.*, 1987), and they combine to form the dimeric enzymes B₁B₁ (Y₁Y₁), B₁B₂ (Y₁Y₄) and B₂B₂ (Y₄Y₄). However, until now the basis for the immunochemical and catalytic differences between subunits B₁ and B₂ has remained unclear. Although subunits B₁ and B₂ yield distinct peptide maps (Stockman *et al.*, 1987), the protein sequencing experiments, which would establish unequivocally the molecular nature of these differences, have not been undertaken previously.

During the present study we have subjected the B₁ and B₂ subunits to amino acid sequence analysis. The data contained in Fig. 4 provide clear evidence that subunits B₁ and B₂ do not arise from post-synthetic modifications. They are the products of separate genes; subunits B₁ and B₂ contain different amino acid residues at positions 88, 110, 111, 112, 116, 124 and 127.

A comparison of the amino acid sequence data for subunit B₁ with DNA sequence data for human GST (Fig. 4) shows complete agreement with the deduced protein sequence of the *GTH*₁ cDNA described by Tu & Qian (1986). Independently, Board & Webb (1987) have cloned the same gene, which they call *GST* 2-3. Hence *GTH*₁ and *GST* 2-3 genes both appear to encode the B₁ subunit. A similar comparison between our amino acid sequence data of the B₂ subunit and other primary structures derived from DNA cloning experiments showed close similarity (Fig. 4), but not complete identity, between subunit B₂ and the protein that would be encoded by the *GTH*₂ clone described by Rhoads *et al.* (1987).

Approx. 50 % of subunit B₂ has been sequenced in this study and it has been found that all but one of the amino acid residues identified were identical with those predicted by the *GTH*₂ cDNA sequence. At this discrepant position, residue 111, *GTH*₂ cDNA possesses the codon ACT, which codes for threonine, whereas subunit B₂ was found to contain serine. It is noteworthy that residue 111 is one of the 11 amino acid residues (out of 221) that differ in the proteins that would be encoded by *GTH*₁ and *GTH*₂ cDNAs. Indeed, as the adjacent residues, 110 and 112, also differ, this region of the two GSTs is particularly variable, with their equivalent codons in *GTH*₁ and *GTH*₂ cDNAs containing five base changes.

It appears likely that this difference at position 111 represents allelic variation. As both serine and threonine have aliphatic hydroxy side chains this putative change is conservative and will not manifest itself by obvious physicochemical differences at the protein level. Despite this difference subunit B₂ and the subunit of the protein encoded by *GTH*₂ cDNA, referred to as GST 2-2, are probably functionally indistinguishable as both GST B₂B₂ and GST 2-2 have been shown to exhibit about 4-fold greater glutathione peroxidase activity, with cumene hydroperoxide as substrate, than GST B₁B₁ and GST 1-1

respectively (Stockman *et al.*, 1987; Chow *et al.*, 1988).

The B₁ and B₂ subunits can be resolved by reverse-phase h.p.l.c., and their elution positions (Fig. 2) suggest that they are equivalent to the α_x and α_y h.p.l.c. peaks that were reported by Ostlund Farrants *et al.* (1987) in their paper describing the use of this method to isolate GST subunits. Hence subunit B₁, subunit Y₁ and α_x h.p.l.c. peak are all equivalent polypeptides and appear to be identical with the subunit encoded by *GTH*₁ cDNA. Similarly subunit B₂, subunit Y₄ and α_y h.p.l.c. peak are all equivalent and are essentially the same as the subunit encoded by *GTH*₂ cDNA.

It is not yet certain how many Alpha class GSTs exist in human liver, but it is generally agreed that three major hepatic GSTs, namely B₁B₁, B₁B₂ and B₂B₂, are members of this family (Stockman *et al.*, 1985; Soma *et al.*, 1986; Sugimoto *et al.*, 1987). The data of Vander Jagt *et al.* (1985) describing the isolation of 13 Alpha class GSTs from human liver are at variance with many other workers in the field. It appears highly likely that the large number of forms recovered by Vander Jagt *et al.* (1985) do not represent separate enzymes but are multiple forms generated by the purification strategy, namely GSH-Sepharose affinity chromatography and chromatofocusing. Indeed, close examination of the properties of these 13 enzymes shows that they can be subdivided into three groups, presumably equivalent to GSTs B₁B₁, B₁B₂ and B₂B₂ (Stockman, 1987). However, in fairness to Vander Jagt *et al.* (1985), it should be noted that the B₁-subunit-containing enzymes have a tendency to be eluted from ion-exchangers as multiple peaks and that this behaviour is influenced by the presence of GSH. For example, Hayes *et al.* (1983) resolved the single enzyme, now called GST B₁B₁, into several isoforms by using CM-cellulose chromatography; these multiple peaks coalesce when 2 mM-GSH is included in the eluting buffer but not when other reducing agents are employed (P. K. Stockman & J. D. Hayes, unpublished work). Also, during purification of renal GSTs Tateoka *et al.* (1987) showed that GST B₁B₁ was eluted from chromatofocusing columns as two peaks at pH 8.7 and 8.5 whereas GST B₁B₂ was eluted at pH 8.2 and 7.7; GSH was not included in the buffer that was used to elute the chromatofocusing columns.

The data presented in Fig. 1 show that whereas all livers contain the B₁B₂ heterodimer the amounts of the B₁B₁ and the B₂B₂ homodimers in different specimens vary considerably. For example, livers 5 and 6 possess little GST B₁B₁ but reasonably large amounts of GST B₂B₂. Conversely, liver 8 possesses predominantly GST B₁B₁. All livers therefore appear to express subunits B₁ and B₂ but the relative amounts of these subunits within the hepatocyte is very variable. Two separate lines of evidence support the contention that all livers express subunits B₁ and B₂ but at different amounts. Firstly, reverse-phase h.p.l.c. analysis of the *S*-hexyl-GSH-Sepharose-affinity-purified GST pools from the liver specimens described by Hussey *et al.* (1986) has shown the B₁/B₂ subunit ratio to vary at least 40-fold, from 4:1 to 1:10 (J. D. Hayes, unpublished work). Secondly, radioimmunoassays have been developed that allow the concentrations of the B₁ and B₂ subunits to be measured in human plasma (Beckett & Hayes, 1984). These radioimmunoassays were developed for clinical studies, but their use in control populations has shown that the

plasma concentrations of subunits B₁ and B₂ vary considerably and, as a consequence, give a rather wide reference range (Beckett & Hayes, 1987). Although both the h.p.l.c. method and the radioimmunoassays reveal large inter-individual variations in the expression of subunits B₁ and B₂ it is equally important to note that neither technique has demonstrated individuals who lack, or are nulled for, either subunit B₁ or subunit B₂. Together, these data indicate that subunits B₁ and B₂ are not allelic variants but are encoded at different gene loci. It must be concluded, however, that the structural genes for subunits B₁ and B₂ are physically closely linked as hybridization *in situ* with the GST-2 cDNA probe, which would be expected to hybridize to both the genes encoding subunits B₁ and B₂, resulted in an unambiguous labelling of the p12 band on the short arm of chromosome 6 (Board & Webb, 1987).

The variable expression of the Alpha class GSTs in man is not widely recognized. However, the B₂B₂ homodimer has a particularly high glutathione peroxidase activity, and it is possible that individuals with low concentrations of subunit B₂ are more susceptible to oxidative stress and lipid peroxidation. Certain antitumour antibiotics such as adriamycin, mitomycin and bleomycin exert their effects through free-radical reactions, and the expression of GST B₂B₂ may influence the effectiveness of these drugs. It can be expected that these inter-individual differences in the expression of subunits B₁ and B₂ represent an important factor in both acquired and intrinsic drug-resistance.

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