Hepatic glutathione S-transferases in mice fed on a diet containing the anticarcinogenic antioxidant butylated hydroxyanisole

Isolation of mouse glutathione S-transferase heterodimers by gradient elution of the glutathione-Sepharose affinity matrix

John D. HAYES,*† Linda A. KERR,† Stephen D. PEACOCK,† Andrew D. CRONSHAW† and Lesley I. McLELLAN* 

*Department of Clinical Chemistry, University of Edinburgh, The Royal Infirmary, Edinburgh EH3 9YW, and †WELMET Protein Sequencing Laboratory, Department of Biochemistry, University of Edinburgh, George Square, Edinburgh EH8 9XD, Scotland, U.K.

Induction of glutathione S-transferases (GSTs) is believed to represent an important mechanism whereby butylated hydroxyanisole inhibits chemical carcinogenesis. The soluble hepatic GSTs expressed by mice fed on normal diets are all homodimers comprising Ya₂ (Mr 25800), Yb₁ (Mr 26400) and Yf (Mr 24800) subunits. In addition to these constitutively expressed GSTs, we have identified enzymes containing Ya₄ (Mr 25600), Ya₄ (Mr 25600), Yb₁ (Mr 26200) and Yb₂ (Mr 26500) subunits from the livers of BALB/c mice fed on diets containing butylated hydroxyanisole (BHA). Gradient affinity elution of GSH-Sepharose has been used to resolve the mouse liver enzymes into several discrete pools of activity from which GSTs were purified by ion-exchange chromatography. The inducible Mu-class Yb₂ and Yb₂ subunits were separately isolated as the heterodimers GST Yb₁Yb₂ and GST Yb₁Yb₂ and their catalytic properties are described; this showed that 1,2-dichloro-4-nitrobenzene and trans-4-phenylbut-3-en-2-one are marker substrates for the mouse Yb₁ and Yb₂ subunits respectively, but no discriminating model substrate was found that allows the identification of the Yb₂ subunit. Individual GST subunits were resolved by reverse-phase h.p.l.c. and their amino acid compositions were determined. Certain subunits (Yb₁, Yb₂ Yb₂ and Yf) were also subjected to automated amino acid sequence analysis, and this demonstrated that the Yb₂ subunit has a blocked N-terminus. The mouse Yb₁, Yb₂ and Yb₂ subunits from the major inducible Mu-class heterodimers were cleaved with CNBr and purified peptides from the Yb₂ and Yb₂ subunits were sequenced. These data show that the Yb₂ subunit is distinct from the GSTs that are encoded by the cDNAs that have been cloned from mouse liver cDNA libraries but possesses identity with the protein that is encoded by pmGT₂, a cDNA isolated from a mouse fibroblast cell line by Townsend, Goldsmith, Pickett & Cowan [(1989) J. Biol. Chem. 264, 21582–21590]. The sequence data also show that the cDNA encoding the mouse Yb₂ subunit has not, to date, been cloned, and the relationship between this subunit and Mu-class GSTs in other species that possess a blocked N-terminus (e.g. rat GST Yoyo) is discussed.

INTRODUCTION

A wide variety of structurally unrelated compounds have been described that help prevent chemical carcinogenesis. These compounds are called chemoprotectors, and include natural dietary components as well as the synthetic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene and ethoxyquin. Although such chemoprotectors exert varied and multiple biological effects, they all induce phase II drug-metabolizing enzymes such as DT-diaphorase, UDP-glucurononitransferases and glutathione S-transferases (GSTs) (Benson et al., 1978, 1980; Wattenberg, 1985; Talalay, 1989). It is believed that, as certain of these enzymes are involved in the detoxication of carcinogens, their over-expression represents the mechanism whereby many chemoprotectors produce resistance to carcinogenesis. The fact that a correlation exists between GST induction and the inhibition of chemical carcinogenesis (Sparrnins & Wattenberg, 1981; Sparrnins et al., 1982) suggests that GSTs are probably of prime importance in the prevention of chemical carcinogenesis.

Most of the toxicological investigations into the effects of chemoprotectors have been performed with the mouse as an experimental model (Wattenberg, 1978; Sparrnins et al., 1982, 1986, 1988), and, as a consequence, several research groups have examined the effects of chemoprotectors on mouse GST expression (Benson et al., 1978, 1989; Pearson et al., 1983; Parchment & Benson, 1984; Benson & Barretto, 1985; McLeellan & Hayes 1989). These studies have shown that, like the rat and human GSTs, the cytosolic forms in the mouse can be subdivided into four groups or classes. Three of these comprise cytosolic enzymes and have been designated Alpha, Mu and Pi (Mannervik et al., 1985), and the fourth is membrane-bound and is called microsomal GST (Morgenstern et al., 1982). The chemoprotector BHA induces members of the mouse Alpha-, Mu- and Pi-class GSTs by significant amounts but has relatively little effect on the microsomal GST (McLeellan & Hayes, 1989). The cytosolic GSTs are dimeric, and in the mouse the Alpha-, Mu- and Pi-class enzymes are composed of Ya-type (Ya₁, Mr 25600 or Ya₂, Mr 25800), Yb₁ (Mr 26400) and Yf (Mr 24800) subunits respectively (McLeellan & Hayes, 1989). By contrast with the Pi-class GST, which probably contains only a single enzyme (GST YfYf), both the Alpha and the Mu classes of GST contain several distinct enzymes.

At the enzyme level, relatively little is known about either the Mu-class or the Alpha-class GSTs that are induced by chemoprotectors. Among the Mu-class enzymes, several laboratories

Abbreviations used: GST, glutathione S-transferase; BHA, butylated hydroxyanisole.

†To whom correspondence should be addressed.
have purified GST Yb1, Yb2, the enzyme that is expressed constitutively in mouse liver (Lee et al., 1981; Agius & Gidari, 1985; Hatayama et al., 1986; Warholm et al., 1986; Hayes et al., 1987). Benson and her colleagues have also isolated this constitutive form (which they designate GT8.7) as well as several additional Mu-class enzymes (GT8.8a, GT8.8b and GT9.3) that were isolated from mice fed on a BHA-containing diet (Benson et al., 1979, 1989; Pearson et al., 1983; Parchment & Benson, 1984). Pearson et al. (1988) have provided evidence that GT8.7 and GT9.3 are genetically distinct by isolating, from the livers of BHA-treated mice, two separate cDNA clones, pGT875 and pGT55, which encode these two GSTs.

More recently, Townsend et al. (1989) have isolated from a mouse fibroblast 1L29 cell line two Mu-class GST cDNA clones, pmGT2 and pmGT10, and, whereas pmGT10 is essentially identical with pGT875, the pmGT2 clone encodes a novel Yb subunit. From their comparisons between the primary structures of the mouse and rat Mu-class GST cDNAs, Townsend et al. (1989) concluded that the subunits encoded by pGT875, pmGT2 and pGT55 are the analogues of the rat Yb1, Yb2 and Yb4 subunits respectively [see Lai et al. (1988) for details of Yb].

In the present study, we describe the gradient affinity elution of GSH-Sepharose to resolve mouse hepatic GSTs. This procedure has been employed as the initial step in the purification of mouse GSTs, and its use has facilitated the isolation of several heterodimers, both Alpha-class and Mu-class, that have not been identified previously. We provide evidence that the major Mu-class enzymes isolated from the livers of mice fed on diets containing BHA are GST Yb1,Yb2 and Yb4,Yb2. An additional Yb-type subunit, which, unlike subunits Yb1 and Yb2, possesses a blocked N-terminus, has been purified; this is referred to as Yb0 and was isolated from the heterodimer Yb1,Yb2.

MATERIALS AND METHODS

Chemicals

These were all of analytical grade and were obtained from the sources reported in previous publications from our laboratories (McLellan & Hayes, 1989; Hayes et al., 1989, 1990). The GSH-Sepharose matrix used for affinity chromatography was prepared by the method of Simons & Vander Jagt (1977).

Animals

Male and female Balb/c mice, which were 8 weeks old, were purchased from Bantin and Kingman, Hull, U.K. These animals were fed on a diet containing 0.75% (by wt.) of BHA for 2 weeks (for further details of the diet see McLellan & Hayes, 1989) before being killed on day 14. The livers were removed and stored at −85°C until required for enzyme purification.

Preparation of cytosol

Livers from male and female mice were processed separately. The frozen mouse livers (usually 60 g of tissue was processed for each preparation) were allowed to thaw at room temperature before being cut into small pieces and blended in 5 vol. of ice-cold 200 mM-NaCl/0.5 mM-dithiothreitol/50 mM-Tris/HCl buffer, pH 7.8 (buffer A). The tissue extract was subjected to an initial centrifugation at 10000 g for 40 min at 4°C, and the resulting supernatant was then centrifuged at 150000 g for 90 min at 4°C. This second supernatant (cytosol) was filtered through a plug of glass Wool before being dialysed at 4°C against three changes, each of 2 litres, of buffer A over 18 h. The dialysed cytosol was re-centrifuged at 10000 g for 60 min at 4°C, to remove precipitated protein, before being applied to the GSH-Sepharose column.

Analytical

Enzyme assays were performed at 37°C by using methods described elsewhere (Habig & Jakoby, 1981; McLellan & Hayes, 1989). Protein determinations were carried out by the method of Bradford (1976).

Discontinuous SDS/PAGE was performed in 16% (w/v) resolving gels comprising 0.09% NN'-methylenebisacrylamide. The gels were calibrated with a mixture of rat liver GSTs containing Ya (M, 25500), Yb (M, 26300) and Yc (M, 27500) subunits. Where possible, purified mouse Yf subunit (M, 24800) was included as an electrophoretic standard. A detailed account of the behaviour of GSTs during SDS/PAGE is to be found elsewhere (Hayes & Mantle, 1986).

Individual GST subunits were identified and prepared by reverse-phase h.p.l.c. (Ostlund Farrants et al., 1987). This was carried out on a Waters 0.39 cm × 30.0 cm μBondapak C18 column (10 μm particle size) developed with a linear 30–60% (v/v) gradient of acetonitrile inaq. 0.1% (v/v) trifluoroacetic acid. Although significant between-batch variations occur in the elution positions of GSTs from the μBondapak column, it was found that the relative order of elution of the different mouse subunits did not change. When replicates were performed within the same batch (over a period of 48 h) a maximum variation of 1 min in the retention time of the GST polypeptides was observed; however, this variation affected all subunits, and it is assumed to represent a non-specific drift (possibly due to changes in ambient temperature). A greater variation in retention times, of as much as 4 min, was noted when samples were re-analysed in the different batches, which may have been performed 15 months apart. However, the analyses of mouse GST subunits that are presented in this paper (Figs. 3, 5 and 6) were all obtained within the same batch, and on this occasion repeat analysis of a calibration mixture (Ya, Yb and Yf) showed that during the run a drift of about 0.8 min occurred in the retention time of the subunits. The retention times quoted in the text represent the mean result of either duplicate or triplicate analyses.

Amino acid sequencing

Samples (either purified GST subunits or peptides obtained after CNBr cleavage) were sequenced with the use of an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser (Warrington, U.K.) as reported previously (Hayes et al., 1989).

EXPERIMENTAL AND RESULTS

The livers of mice fed on normal diets contain Ya,Ya, Yb,Yb, and YYf dimers. These enzymes have been investigated by several groups (Hatayama et al., 1986; Warholm et al., 1986; Hayes et al., 1987; McLellan & Hayes, 1987; Benson et al., 1989), and the present work indicates that, collectively, the Ya, Yb and Yf subunits account for 98% of the hepatic GSTs in control mice. During the present study of the hepatic GSTs in BHA-fed mice three separate purifications were undertaken, each of which gave essentially identical results.

Isolation of mouse GSTs by affinity chromatography

Columns (1.6 cm × 45 cm) of GSH-Sepharose that had been equilibrated with buffer A were typically found to retain 90–95% of the 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity in hepatic cytosol from BHA-fed mice. The amount of GST activity eluted in the ‘flow-through’ fractions did not vary with sex, and the enzymes that did not bind to GSH-Sepharose were not characterized, as they have been studied previously (McLellan & Hayes, 1989).
After application of mouse liver cytosol to the columns of GSH-Sepharose, the columns were each washed with about 500 ml of buffer A to remove protein that was bound nonspecifically. The affinity matrix was developed in two stages by two separate linear gradients of GSH, both of which were of 400 ml and were formed in 200 mM-Tris/HCl buffer, pH 9.3. The first, a shallow gradient of 0–2.5 mM-GSH, eluted two discrete peaks of GST activity; these were recovered in fractions 11–13 and 18–28. Similarly, the second, a steeper gradient of 2.5–50.0 mM-GSH, also eluted two peaks of activity; these were recovered in fractions 60–67 and 70–80. With this two-stage elution strategy base-line separation was not obtained between the peaks of activity eluted in fractions 18–28 and that eluted in fractions 60–67. Indeed, an ill-defined and broad protein peak can be discerned in Fig. 1 that was recovered in fractions 38–50. Therefore, as Fig. 1 indicates, the mouse hepatic protein eluted from the GSH-Sepharose matrix was divided into five pools.

The peaks of GST that were resolved by gradient affinity elution possessed markedly different catalytic properties (see Fig. 1). All peaks were active with 1-chloro-2,4-dinitrobenzene; pools 1–5 had specific activity towards 1-chloro-2,4-dinitrobenzene of 10.0, 12.0, 56.4, 72.7 and 180.2 nmol/min per mg respectively. Pool 2 (fractions 18–28) had the highest peroxidase activity towards cumene hydroperoxide; pools 1–5 had specific activities towards this compound of 1.6, 29.8, 0.4, 0.3 and 0.05 nmol/min per mg respectively. Most of the GST activity with ethacrynic acid was eluted with pool 4, fractions 60–67 (results not shown), and that towards 1,2-dichloro-4-nitrobenzene was associated with pool 5 (fractions 70–80). As cumene hydroperoxide, ethacrynic acid and 1,2-dichloro-4-nitrobenzene are relatively specific for the mouse Ya, Yf and Yb, subunits respectively, the elution profile suggests that these subunits are eluted primarily in pools 2, 4 and 5 respectively. Besides pool 5, a significant amount of 1,2-dichloro-4-nitrobenzene–GSH-conjugating activity was also recovered in pool 4. By analogy with the elution profile of rat GSTs from GSH–Sepharose (Hayes, 1990), it was considered likely that the activity in pool 4 towards 1,2-dichloro-4-nitrobenzene was due to the presence of a mouse Yb-containing heterodimer.

SDS/PAGE was undertaken to help establish the purity of the GST peaks obtained from the affinity column (Fig. 2). Comparison of the electrophoretic mobilities of these enzymes with the rat Ya (M, 25500), Yb (M, 26300) and Yc (M, 27500) polypeptides as well as with a preparation of the mouse GST Yf subunit indicated that the constitutively expressed Ya, Yf and Yb, subunits are recovered in pools 2, 4 and 5 respectively; this result is consistent with the substrate-specificities of these pools, as described above.

The data presented in Figs. 1 and 2 were obtained with livers from female Balb/c mice. When livers from male Balb/c mice were used as the enzyme source, closely similar results to those shown in Figs. 1 and 2 were obtained (results not shown). In this context it may be noted that in male mouse liver GST YfYf is expressed constitutively at high levels but its expression does not appear to be increased by BHA. By contrast, GST YfYf is not expressed to a great extent in the livers of female mice fed on a normal diet (approx. 15-fold less than in males), but, as can be concluded from the fact that the elution profiles of hepatic GSTs from male and female BHA-fed mice are indistinguishable, subunit Yf is induced in female mice by BHA to those levels found in male livers. A comparable sex-specific induction of the Yf subunit has also been observed in 129/OLA mice (McLellan & Hayes, 1989).

Purification of Alpha-class GSTs

Pools 1 and 2 eluted from the GSH-Sepharose column by the GSH gradient were combined separately (see Fig. 1). As SDS/PAGE indicated that both these pools were highly purified, they were subjected to reverse-phase h.p.l.c. on a µBondapak column, as described in the Materials and methods section, to
provide an independent assessment of the number of GST subunits present. The chromatograms obtained (Fig. 3) demonstrated that pool 1 contains a complex mixture of polypeptides whereas pool 2 represents an essentially homo-

Fig. 2. Electrophoretic analysis of hepatic GSTs from BHA-fed mice

The five pools of GST activity that were obtained by gradient elution from GSH-Sepharose (see Fig. 1) were subjected to SDS/PAGE. Samples (4 μg of protein) from each of the five pools were analysed in 16% (w/v) polyacrylamide gels containing 0.09% (w/v) N,N'-methylenebisacrylamide. The samples were applied to the gel, from left to right, as follows: track 1, rat liver Ya (Mr 25 500), Yb (Mr 26 300) and Yc (Mr 27 500); track 2, mouse pool 1; track 3, mouse pool 2; track 4, mouse pool 3; track 5, mouse pool 4; track 6, mouse pool 5; track 7, rat liver Ya, Yb and Yc subunits; track 8, mouse Yf subunit.

Purification of Pi-class GSTs

The Pi-class enzyme, GST YfYf, was recovered in pool 4 (fractions 60–67) from the GSH-Sepharose column. However, pool 4 contains not only Yf subunits but also at least two other polypeptides with a slower electrophoretic mobility (Fig. 2). To purify GST YfYf in pool 4, the protein was dialysed at 4°C against four changes, each of 2 litres, of 0.5 mm-dithiothreitol/10 mm-sodium phosphate that had been adjusted to pH 6.0 with orthophosphoric acid (buffer B), before being subjected to cation-exchange chromatography. This was carried out at room temperature on a Protein PAK Glass SP-5PW column (0.8 cm x 7.5 cm) that had been equilibrated with buffer B and was developed with a 0–400 mM-NaCl gradient; chromatography was undertaken in a Waters Advanced (650E) Protein Purification System. The SP-5PW column resolved pool 4 into four protein-containing peaks (Fig. 4), which were eluted at 8–12 min, 35–40 min, 46–48 min and 50–60 min. The two peaks eluted at 8–12 min (4a) and 35–40 min (4b) appeared to be interconvertible during chromatography; during certain preparations peak 4a disappeared and peak 4b was found to contain a correspondingly greater amount of material. SDS/PAGE demonstrated that the first two peaks (4a and 4b) only contained Yf subunits whereas the latter two (4c and 4d) contained subunits with a similar mobility to the rat Yb-type subunit. Analysis of the two Yf-containing enzyme preparations (4a and 4b) by reverse-phase h.p.l.c. showed that they were both at least 95% pure. These two preparations yielded closely similar chromatograms and the elution profile obtained from 4b is shown in Fig. 5.

Fig. 3. Reverse-phase h.p.l.c. analysis of Alpha-class GSTs

Hepatic GSTs from mice fed on a diet containing BHA were resolved by gradient elution from GSH–Sepharose as described in Fig. 1. Fractions 11–13 and 18–28 from the affinity column were combined separately and are referred to as pool 1 (a) and pool 2 (b) respectively. Portions of these two pools were subjected to reverse-phase h.p.l.c. on a 0.39 cm x 30.0 cm μBondapak C18 column developed with a linear 30–60% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid. The eluate was monitored at 220 nm. During the first 5 min of each run, as the sample was loaded isocratically on to the column, the flow rate was increased linearly from 0.1 ml/min to 1.0 ml/min and thereafter the flow rate was maintained at 1.0 ml/min. The relative output of pump B is shown by the continuous straight line; pump A delivered 30% acetonitrile and pump B delivered 60% acetonitrile.
Inducible mouse glutathione S-transferase subunits

**Fig. 5. Reverse-phase h.p.l.c. of Pi-class GST**

The Pi-class GST, which was eluted from the GSH–Sepharose affinity column in pool 4 (see Fig. 1), was purified by cation-exchange chromatography on a Protein PAK Glass SP-5PW column, as shown in Fig. 4. The material eluted from the cation-exchanger between 35 and 40 min was combined and is referred to as pool 4b. This fraction migrated as a single band of Mr 24800 during SDS/PAGE and was subjected to analysis by h.p.l.c. to assess its purity. Reverse-phase h.p.l.c. was performed on a μBondapak C18 column as described in the legend to Fig. 3.

**Purification of Mu-class GSTs**

Members of this class of enzyme were eluted from GSH–Sepharose, by the GSH gradient, in pools 3, 4 and 5 (see Fig. 1). Quantitatively, the GST in pool 3 did not represent a major isoenzyme and it was therefore not characterized further during the present study. As shown in Fig. 2, SDS/PAGE revealed that pool 4 contained at least two Yb-type subunits of Mr 26400 and Mr 26200, whereas pool 5 contained a single Yb-type subunit of Mr 26400. The GST eluted in pool 5 represents the constitutively expressed Mu-class GST Yb\textsubscript{b}.

In addition to the Mu-class GSTs, pool 4 also contains GST Yb\textsubscript{YYF}. The Mu- and Pi-class GSTs were prepared separately by cation-exchange chromatography on a Protein PAK Glass SP-SPW column, as described above (Fig. 4). Two Mu-class GSTs, 4c and 4d, were resolved by this step and were eluted at 46–48 min and 50–60 min respectively. However, the 4c peak, by comparison with the 4d peak, represents a relatively minor enzyme, accounting for about 15% of the Mu-class GSTs in pool 4.

SDS/PAGE of the 4c and 4d preparations indicated that they both represent distinct heterodimeric enzymes. The two preparations appeared to contain a common subunit that co-migrated during electrophoresis with the constitutive Yb\textsubscript{b} subunit of Mr 26400. However, the additional electrophoretic band in 4c has an estimated Mr of 26500, whereas the additional electrophoretic band in 4d has an estimated Mr of 26200.

Reverse-phase h.p.l.c. of the Mu-class enzymes was undertaken to assess purity and to assist in the identification of the Yb-type subunits. This demonstrated that the Mu-class GSTs recovered in pools 4c, 4d and 5 are highly purified (Fig. 6). The constitutively expressed Mu-class GST Yb\textsubscript{b} was eluted from the μBondapak column, by the acetonitrile gradient, as a single peak with a retention time of about 38 min. The GST subunits in the 4c pool were eluted from the μBondapak column as two major peaks at 38.5 min and 56 min; pool 4c also contained small amounts of a minor polypeptide that was eluted at 45 min and usually accounted for less than 5% of the protein in the 4c preparation (amino acid sequencing showed that this material represents contaminating Yf subunit). The GST 4d preparation was resolved by reverse-phase h.p.l.c. into two polypeptides that were eluted from the μBondapak column at 38 min and 41 min.

SDS/PAGE of the individual peaks obtained by reverse-phase h.p.l.c. was undertaken to confirm their identity. Fig. 7 shows that the subunit in pools 4c, 4d and 5 eluted from the μBondapak column between 38 and 38.5 min possessed identical electrophoretic mobilities during SDS/PAGE; this subunit, which was subsequently identified as Yb\textsubscript{b}, has a closely similar mobility during SDS/PAGE to the rat Yb (Mr 26300) standard. Fig. 7 also shows that the subunit in pool 4d eluted from the h.p.l.c. column at 41 min (Yb\textsubscript{b}) has a greater electrophoretic mobility than that eluted at approx. 38 min (Yb\textsubscript{b}). Moreover, the subunit in pool 4c eluted at 56 min (Yb\textsubscript{b}) has a lower mobility during SDS/PAGE than the subunit eluted between 38 and 38.5 min. From these data it was estimated that the Yb\textsubscript{b} subunits have Mr values of 26400, 26200 and 26500 respectively.

**Catalytic properties of hepatic Mu-class GSTs**

The catalytic properties of these Yb-containing GSTs were compared and Table 1 shows that they are functionally distinct. It should be noted that all the preparations, pools 4c, 4d and 5, possess activity towards 1,2-dichloro-4-nitrobenzene, which is consistent with the hypothesis that they all contain the Yb\textsubscript{b} subunit. As expected, the Yb\textsubscript{b} homodimer (pool 5) has approx. 2-fold greater specific activity towards 1,2-dichloro-4-nitrobenzene than the other Mu-class GSTs, which is consistent with its quaternary structure. Interestingly, GST Yb\textsubscript{b}, Yb\textsubscript{b}, has
Hepatic Mu-class GSTs from mice fed on a diet containing BHA were prepared as described in the text. The two Mu-class heterodimers, 4c and 4d, were purified from the GST pool 4 recovered in fractions 60–67 obtained from GSH-Sepharose (see Fig. 1). This material was subjected to cation-exchange chromatography as described in Fig. 4 and the GSTs eluted at 46–48 min and 50–60 min are referred to as 4c and 4d respectively.

Fig. 6. Resolution of mouse GST subunits by reverse-phase h.p.l.c.

Portions of GST 4c (a) and GST 4d (b) were subjected to reverse-phase h.p.l.c., as described in Fig. 3. To permit identification of the Yb subunit, pool 5 (see Fig. 1), which represents GST YbYb, was also examined by reverse-phase h.p.l.c. and the elution profile of Yb, Yb, from the aBondapak column is shown in (c). During this project three separate batches of the three Mu-class GSTs were prepared and all were examined by reverse-phase h.p.l.c. The chromatograms shown in (a)-(c) represent typical examples of the results obtained for these enzymes; for further details about between-batch variation see the Materials and methods section.

significantly greater activity towards 1-chloro-2,4-dinitrobenzene than the other Mu-class GSTs, which suggests that the Yb and Yb subunits exhibit little activity towards this compound.

The 4d enzyme, which contained the subunit of M, 26200, possesses a significantly greater activity towards trans-4-phenylbut-3-en-2-one (5-fold) than the other Mu-class GSTs. As this substrate is specific for the Yb subunit in the rat (Hayes, 1984), the catalytic results in Table 1 suggest that the mouse GST polypeptide of M, 26200 is closely related to the rat Yb subunit. No marker activity was found for the 4c enzyme preparation, and therefore we can suggest no diagnostic substrate for the subunit of M, 26200.

It should also be noted that the specific activities of YbYb, contained in Table 1 are in close agreement with those published previously (Hayes et al., 1987; McLellan & Hayes, 1987), despite being obtained from batches prepared more than 3 years apart.

**N-Terminal sequence analysis of mouse Mu-class GST subunits**

The subunits purified from the Mu-class GST 4c and 4d preparations by reverse-phase h.p.l.c. were subjected to automated amino acid sequencing to establish their relationship with GST YbYb. The data in Fig. 8 support the supposition that the subunit designated Yb, which was obtained from both the GST 4c and 4d preparations (it was eluted from the aBondapak column between 38 and 38.5 min; see Fig. 6), is identical with the constitutively expressed hepatic Mu-class GST subunit. The N-terminal amino acid sequence of subunit Yb (which has a retention time of 41 min from the aBondapak column) demonstrates that it possesses a distinct primary structure from subunit Yb. Over the 32 N-terminal amino acid residues for which we obtained sequence data, a comparison between subunits Yb and Yb revealed ten substitutions, at residues 4, 9, 10, 14, 16, 19, 26, 29, 30 and 32. The subunit from GST preparation 4c eluted from the aBondapak C18 column at 56 min was found to possess a blocked N-terminus.
Table 1. Specific activities of Mu-class GSTs with selected substrates

The GST 4c and 4d preparations were purified by cation-exchange chromatography as shown in Fig. 4. The Mu-class GST Yb1Yb3 was obtained by gradient affinity elution of GSH-Sepharose in the fractions designated pool 5 (see Fig. 1). The subunit compositions of the different enzymes are defined by their primary structures (see Figs. 8, 9 and 11). The enzymes assays were all performed at 37 °C as outlined in the Materials and methods section. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, trans-4-phenylbut-3-en-2-one. All results are expressed as means ± s.d. for four determinations. The activities of GSTs prepared from two separate batches were examined and these gave closely similar results. However, the data presented are results obtained from a single batch.

<table>
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<tr>
<th>Pool from affinity column</th>
<th>$10^{-3}\times M_r$ of GST subunits</th>
<th>GST subunit composition</th>
<th>Specific activity (μmol/min per mg)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CDNB</td>
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<tr>
<td>4c</td>
<td>26.4, 26.5</td>
<td>Yb1Yb3</td>
<td>74 ± 4</td>
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<td>4(d)</td>
<td>26.4, 26.2</td>
<td>Yb1Yb2</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>26.4, 26.4</td>
<td>Yb1Yb1</td>
<td>193 ± 8</td>
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Fig. 8. N-Terminal amino acid sequence analysis of mouse GST subunits

GST preparations 4c and 4d were obtained as described in the text and the individual subunits prepared by reverse-phase h.p.l.c. The retention times from the µBondapak column are each mean values of triplicate analyses, which were all performed within the same batch for the purpose of automated amino acid sequencing. Amino acid sequence analysis was performed as described previously (Hayes et al., 1989). Two of the mouse Mu-class polypeptides were called Yb1 and Yb2 on the basis of sequence similarity to the rat GST subunits (for further details see Townsend et al., 1989). The subunit from GST 4c that is designated Yb2 is believed to have a blocked N-terminus, as no phenylthiohydantoin-derivatives were obtained after automated Edman degradation. The system of numbering the amino acid residues includes the initiator methionine, and therefore the N-terminal residue (proline) in the mature Yb1, Yb2, and Yf subunits is designated residue 2. The amino acids marked by an asterisk (*) are those that differ between the Yb-type polypeptides. The Pi-class Yf subunit, which was isolated as a minor contaminant in the GST 4c preparation (see Fig. 6a), is included for comparison; the N-terminal sequence of subunit Yf is in agreement with that reported by Phillips & Mantle (1991) but differs at positions 12, 13 and 14 from that described by Mannervik et al. (1985).

Table 2. Amino acid compositions of mouse GST subunits

The amino acid compositions of individual polypeptides resolved by reverse-phase h.p.l.c. were determined with an Applied Biosystems A420 Derivatizer.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Subunit</th>
<th>Yb1</th>
<th>Yb2</th>
<th>Yf</th>
<th>Yb3</th>
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Fig. 9. Comparison of the primary structure of the mouse Yb₂ subunit with cloned Mu-class GST

Sequence of CNBr-derived peptides from Yb₂ is shown aligned with analogous sequences deduced from the cDNA clones pmGT2 (Townsend et al., 1989), pGT875a and pGT55a (Pearson et al., 1988). The residues marked by an asterisk (*) are those that differ between the proteins that would be encoded by the three cDNA clones. It should be noted that the system of numbering the residues includes the initiator methionine, which is not represented in the mature protein.

Amino acid compositions of GST subunits

The amino acid compositions of those subunits that had a blocked N-terminus (i.e. Ya-type and Yb₂) were determined to help establish their relationship to other GSTs. Table 2 shows that subunits Ya₁ and Ya₃ have similar amino acid compositions, and presumably belong to the same gene family, but subunit Yb₃ is distinct from both the Ya-type subunits and the Yf subunit; between these three subunit types major differences were noted in the recoveries of lysine, threonine, serine, glycine and tyrosine.
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Fig. 10. Analytical and preparative maps of the mouse Yb₁, Yb₂, and Yb₅ subunits

The Yb₁, Yb₂ and Yb₅ subunits were separately purified from the heterodimeric enzymes, 4c and 4d, by reverse-phase h.p.l.c. as shown in Fig. 6. After freeze-drying, the subunits were resuspended inaq. 70% (v/v) formic acid before being cleaved with CNBr. The resulting CNBr-cleavage fragments were freeze-dried, redissolved inaq. 0.1% (v/v) trifluoroacetic acid and applied to a Waters µBondapak C₁₈ column (10 µm particle size; 0.39 cm x 30 cm) that had also been equilibrated withaq. 0.1% (v/v) trifluoroacetic acid. This column was developed with a 0–70% (v/v) acetonitrile gradient inaq. 0.1% (v/v) trifluoroacetic acid. The continuous straight line represents the output of pump B, which delivered 70% acetonitrile. Panels (a), (b) and (c) show the chromatograms obtained with the digests of the Yb₁, Yb₂ and Yb₅ subunits respectively.

Primary structures of Yb₁, and Yb₅ subunits

As the GST preparation 4d (Yb₂,Yb₅) represents the major mouse Mu-class heterodimer to be isolated, it was considered desirable to carry out a more extensive structural analysis of the two polypeptides it comprises. We wished to establish whether this new GST represents the hybrid between the subunits encoded by pGT875 (Yb₁) and either pmGT2 (Yb₂) pGT55 (Yb₅) or a novel Yb-type subunit; it should, however, be noted that the N-terminal sequence data (Fig. 8) support the hypothesis that the subunit eluted from the µBondapak C₁₈ column at 41 min represents the GST encoded by pmGT2. A comparison between pGT875 and pmGT2 revealed that, although they show 80% sequence identity, they differ in the number of methionine residues that they encode. The protein produced by pGT875 contains 11 methionine residues (including the initiation codon) whereas that encoded by pmGT2 contains only nine. Of these, eight are common to both proteins and are found at positions 1, 3, 35, 105, 113, 135, 198 and 212. The three additional methionine residues encoded by pGT875 are found at positions 19, 109 and 169, and the remaining unique methionine encoded by pmGT2 is at position 185 (Pearson et al., 1988; Townsend et al., 1989). To allow further structural analysis, subunit Yb₁ and the putative Yb₂ subunit were prepared separately from GST preparation 4d by reverse-phase h.p.l.c. as described in the legend to Fig. 6; multiple injections were carried out and the eluate containing the two subunits was collected manually. The polypeptides thus purified were subjected to CNBr cleavage. Analysis of the digests by reverse-phase h.p.l.c. (see below) revealed major differences in the peptides yielded by subunit Yb₁ and the putative Yb₂ subunit. Sequence analysis of the CNBr-cleavage peptides from the subunit designated Yb₂ show agreement with the protein structure predicted from pmGT2 (Fig. 9), thereby confirming its identity. We obtained sequence information for the Yb₂ subunit from seven of the nine expected CNBr-cleavage peptides. Sequence data have been obtained for 38 of the 55 ‘difference’ residues that are predicted by the cloned mouse cDNAs, pGT875, pGT55 and pmGT2, and at each position subunit Yb₂ is identical with that encoded by pmGT2.

Comparison between Yb₂ and other Mu-class GST subunits

Unlike most Mu-class GST subunits, subunit Yb₂ possesses a blocked N-terminus (see Fig. 8). Immunoblotting experiments with antibodies raised against rat GSTs Yb₁,Yb₂,Yb₅ and YoYo (for definitions see Hayes, 1988) revealed that the mouse Yb₂ subunit is closely related immunochemically to the rat Yo subunit. Interestingly, the Yo subunit is the only rat Mu-class GST that has been shown to have a blocked N-terminus (Kispert et al., 1989).

In order to obtain information about the primary structure of subunit Yb₂ it was necessary, because of the blocked N-terminus, to cleave this polypeptide with CNBr before subjecting individual purified peptides to automated amino acid sequencing. A comparison between the h.p.l.c. elution profiles of the CNBr-cleavage peptides obtained from subunit Yb₂ and those obtained from subunits Yb₁ and Yb₅ (the digests were carried out in parallel) is shown in Fig. 10; these ‘maps’ revealed marked differences in the distribution of methionine residues within the three Mu-class subunits. The sequencing experiments confirmed that major differences exist between the primary structures of subunit Yb₂ and the Yb₁ and Yb₅ subunits. However, over the region analysed (see Fig. 11) subunit Yb₂ was found to possess significant degrees of sequence identity with the rat Yo subunit (Kispert et al., 1989) and the predicted amino acid sequence derived from the cDNA HTGT-6, which encodes a human Mu-class GST with a pI value of 5.2 (Campbell et al., 1990).
DISCUSSION

Identity of mouse GST subunits

Most of the studies into the anticarcinogenic effects of chemoprotectors have been performed with the mouse as an animal model, but relatively little is known about the inducible mouse GSTs [see Prochaska & Talalay (1988) and Talalay et al. (1988)]. Although the livers from mice fed on normal diets express essentially only Ya, Yb, and Yf GST subunits (Hayes et al., 1987; McLellan & Hayes, 1987, 1989), we have described in this paper the isolation of Ya, Ya, Ya, Yb, Yb, Yb, and Yf polypeptides from the livers of mice administered dietary BHA. The fact that subunits Ya, Ya, Ya, and Ya are not recovered in significant amounts from normal mouse liver indicates that their expression is dramatically increased (at least 20-fold) by BHA, a conclusion that, in the case of subunits Ya, Ya, and Ya, is supported by Northern blotting experiments (Pearson et al., 1988; Townsend et al., 1989). In the present study particular attention has been given to the inducible Mu-class GSTs; the molecular characterization of the Alpha-class Ya, Ya, and Ya subunits is described elsewhere (McLellan et al., 1991).

Following the report by Townsend et al. (1989), which showed that the proteins encoded by mouse cDNAs pGT875, pmGT2 and pGT55 possess 93%, 95% and 95% sequence identity respectively with the rat Yb, Yb, and Yb subunits, we determined to use a nomenclature for the mouse GSTs that reflected this inter-species structural relationship. Consequently, the mouse GST isoenzymes that we have purified have been defined by their quaternary structure, using subunit designations that indicate their relationship to rat GST polypeptides. The sequence data shown in Fig. 8 and 9 indicate that the mouse subunits we have designated Yb, Yb, and Yb represent the proteins encoded by the cDNA clones pGT875 and pmGT2, which were isolated respectively by Pearson et al. (1988) and Townsend et al. (1989). The mouse Mu-class subunit that has a blocked N-terminus has been designated Yb since it is distinct from the mouse Yb, Yb, and Yb polypeptides (Townsend et al., 1989) and shares little sequence similarity to the rat Yb subunit described by Abramowitz & Listowsky (1987). It was apparent during the early stages of this project that the mouse Yb subunit is structurally related to the rat Yb subunit, since both polypeptides have similar Mr values, are eluted at similar positions from the μBondapak C18 h.p.l.c. column and possess a blocked N-terminus, and, moreover, subunit Yb was found to cross-react strongly with antibodies raised against rat GST YoYo. Kispert et al. (1989) reported that the primary structure of about 50% of the rat Yb subunit, and comparison of these data with those obtained for subunit Yb, shows that both subunits share substantial sequence identity. As Fig. 11 demonstrates, subunits Yb, Yb, and Yb are both related to the human GST subunit encoded by the cDNA HTGT-6, which also possesses a blocked N-terminus (Campbell et al., 1990).

Value of gradient affinity elution of GSH–Seharose during purification of mouse GSTs

A key feature of our purification scheme was the use of GSH gradients to develop the GSH–Sepharose affinity matrix. This step proved invaluable, as it allowed the Yb,Yb homodimer (pool 5) to be separated from the other Mu-class enzymes at an early stage during the purification, thereby preventing Yb,Yb from either obscuring other Yb-type subunits or from physically saturating chromatography columns.

It is noteworthy that other workers (e.g. Di Simplicio et al., 1991).
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1989) who have elicited mouse liver GSTs from affinity columns in a single step have not succeeded in identifying the heterodimers described in the present study. By using gradient elution from GSH-Sepharose, the Mu-class GSTs were recovered primarily in pools 4 and 5 from the affinity column. GST Yb1Yb4 was eluted separately in pool 5, and the two heterodimers, Yb4Yb4 and Yb2Yb2, were eluted in pool 4; these two enzymes were purified subsequently by cation-exchange chromatography. The catalytic properties of the three Mu-class enzymes isolated in this study differed. GST Yb1Yb4 had the highest activity towards 1,2-dichloro-4-nitrobenzene of all the enzymes examined, indicating that among the GST subunits only Yb4 is active with this substrate. The Yb2Yb4 heterodimer possessed significantly more activity for trans-4-phenylbut-3-en-2-one than did any of the other enzymes, indicating that the Yb2 subunit was responsible for this activity. Although we were unable to identify any substrate that might be characteristic for the Yb2 subunit, this result is not surprising as both Kispert et al. (1989) and Campbell et al. (1990) have emphasized the low activity towards model substrates of the rat and human GSTs that show sequence similarity to mouse subunit Yb2.

Besides facilitating the purification of Mu-class GSTs the gradient affinity elution of GSH-Sepharose also allowed the inducible Alpha-class heterodimers, Ya1Ya1 and Ya2Ya2, to be isolated separately from the constitutive Alpha-class GST Ya1Ya2, McLellan & Hayes (1989, 1990) have shown previously that the mouse Ya1 and Ya2 subunits fail to bind to GSH-Sepharose, and therefore it is concluded that the recovery of these two polypeptides in the ‘pool 1’ eluted from the affinity column is a consequence of hybridization with the Ya2 subunit. It is evident from the fact that the Ya1Ya1 and Ya2Ya2 heterodimers are eluted from GSH-Sepharose earlier than GST Ya1Ya2 that the affinity of these Alpha-class heterodimers for the matrix is significantly lower than that of the Ya1Ya1 homodimer. Unfortunately, our attempts to resolve GST Ya1Ya1 from GST Ya1Ya2 have been unsuccessful and we have therefore been unable to study these two Alpha-class heterodimers individually. However, the present study has demonstrated that reverse-phase h.p.l.c. can be employed to prepare the Ya1, Ya2 and Ya3 subunits separately.

Relationship between GSTs isolated in the present study and those described by other workers

Benson et al. (1989) have isolated seven hepatic GSTs (GT8.7, GT8.8a, GT8.8b, GT9.0, GT9.3, GT10.3 and GT10.6) from mice fed on BHA-containing diets, but, as these workers have used a nomenclature to describe the mouse GSTs that is based on pi values, comparison between our data is made more difficult. From the description by Benson et al. (1989), GT7.8, GT8.7, GT8.8a and GT8.8b appear to be isoforms of GST Yb1Yb4 whereas GT9.3 represents GST Yb1Yb2. The enzyme designated GT9.0 is GST Yb1Yf. GT10.6 represents GST Ya1Ya2 and GT10.3 probably comprises a mixture of all three Ya-type subunits. From the above comparison, it is apparent that Benson and her colleagues have purified neither GST Yb1Yb4 nor GST Yb2Yb4. Conversely, during our study we have not succeeded in identifying Yb1Yf. As Benson et al. (1989) obtained their GST preparations from CD1 mice whereas we have isolated GSTs from Balb/c mice, strain differences may account for these variations in enzyme recovery. In any event, it is clear that mouse GSTs are substantially more complicated than was hitherto believed.

Mouse GSTs, like those in other species, are subject to tissue-specific expression. Lee (1982) isolated a GST from mouse testis that was called Ft and appears to be a Mu-class enzyme. Unfortunately, structural studies of this GST were not undertaken, and, although it is difficult to be certain of its identity, it is possible that it represents a Yb1- or a Yb2-containing enzyme.

Specificity in chemoprotection provided by GST

Different chemoprotectors appear to have widely different effects on the levels of the various GST subunits. Examination of the reverse-phase h.p.l.c. analysis of GST subunits induced by the anticarcinogens BHA and bisethylxanthogen, which was carried out by Benson et al. (1989), indicates that, whereas BHA induces most GST subunits, bisethylxanthogen only induces subunit Yb2 and/or subunit Yb4 (it appears probable that subunits Yb4 and Yb2 are co-eluted from reverse-phase h.p.l.c. columns). It would be interesting to learn whether specificity in the induction of individual GST subunits by particular chemoprotectors is reflected in the level of protection provided against different carcinogens. For example, the Mu-class GSTs are effective at catalysing the conjugation of GSH with the mutagens styrene oxide, trans-stilbene oxide, 1-nitropyrene oxide and 9,10-epoxy-7,8-dihydroxybenzo[a]pyrene (Ketterer, 1988; Stanley & Benson, 1988) as well as catalysing the denitrosation of compounds such as the anticancer drug 1,3-bis-(2-chloroethyl)-1-nitrosourea (Smith et al., 1989) and the DNA-methylating carcinogen 1-methyl-2-nitro-1-nitosoguanidine (Jensen & Mackay, 1990). It is therefore reasonable to assume that variations in the ability of chemoprotectors to induce particular Mu-class isoenzymes will result in differences in the extent of protection provided against some of these compounds.

During the present study we have isolated the Mu-class GST Yb1Yb4 and Yb2Yb4 heterodimers from the livers of mice fed on BHA. The Yb2 subunit does not appear to be present in control mouse livers, and circumstantial evidence suggests that its induction by BHA is likely to be of considerable importance in chemoprotection. Glatt et al. (1983) have shown that the rat Yb2 subunit is able to inactivate the K-region epoxide 5,6-epoxy-benz[a]anthracene and the diol epoxide t-10,11-epoxy-8,9,10,11-tetrahydro-r-8,9-dihydroxybenz[a]anthracene. Our amino acid sequencing data indicate that mouse subunit Yb2 is closely related to the rat Yb2 subunit and it is therefore reasonable to assume that the mouse Yb2 subunit can provide protection against the same spectrum of toxic compounds as rat subunit Yb2. Induction of subunit Yb2 in mouse liver by antioxidants, such as BHA, would therefore appear to represent an important protective mechanism against chemical insult by polycyclic aromatic hydrocarbons. The role of the Yb2 subunit in providing protection against noxious chemicals requires further study.

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