Characterization of a novel glutathione *S*-transferase isoenzyme from mouse lung and liver having structural similarity to rat glutathione *S*-transferase 8–8

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In mouse lung, glutathione *S*-transferase (GST, EC 2.5.1.18) isoenzymes belonging to the three major known classes, Alpha, Mu and Pi, have been previously characterized, along with an isoenzyme (pI 5.7) that could not be identified with the Alpha, Mu or Pi classes of GSTs. In the present studies we have demonstrated that this isoenzyme is also expressed in liver. Its structural, kinetic, and immunological properties have been determined and compared with those of the three classes of GSTs. GST 5.7 has a subunit molecular mass of 23 kDa, which is intermediate between that of the previously characterized Alpha (25 kDa) and Pi (22.5 kDa) class GST subunits of mouse lung. Comparison of peptide maps of GST 5.7 with those representative of Alpha, Mu and Pi class GST isoenzymes of mouse lung showed that it had a distinct peptide fragmentation pattern. Kinetic and immunological properties of GST 5.7 were also distinct from other mouse GST isoenzymes belonging to the Alpha, Mu or Pi classes. *N*-Terminal amino-acid-sequence analysis of a 6 kDa fragment generated by CNBr digestion of mouse lung GST 5.7 revealed a 15-residue sequence that was distinct from sequences of known Alpha, Mu and Pi class mouse GSTs. The sequence, however, matched with the sequence of rat GST 8–8 between amino acid residues 106 and 120 with a 73 % identity. The 6 kDa and 12 kDa fragments generated by CNBr digestion of mouse liver GST 5.7 also gave sequences which matched with those of rat GST 8–8 between positions 106 and 120 and 167 and 186, with a high degree of identity. These studies suggest that mouse GST 5.7 structurally corresponds to rat GST 8–8 and belongs to the Alpha class.

INTRODUCTION

One of the major contributors towards cellular defense mechanisms are a family of isoenzymes collectively called glutathione *S*-transferases (GSTs, EC 2.5.1.18) because of their ability to catalyse the conjugation of a wide variety of xenobiotics and toxicants to glutathione [1,2]. Most cytosolic GST isoenzymes can be grouped into three species-independent classes, Alpha, Mu and Pi, on the basis of their structural, immunological, kinetic and physicochemical properties [3,4]. Multiple isoenzyme forms have been reported in most mammalian tissues investigated [3,4], with considerable microheterogeneity among them [5,6]. The expression of certain GST isoenzymes has been proposed to be tissue-specific [7,8], which may enable individual tissues to meet their specific detoxication needs. Using genetic and recombinant DNA techniques, multiple genes corresponding to the Alpha and Mu classes have been identified for human and rat GST isoenzymes [9–11]. Since recent reports have implicated Pi class GST as a tumour marker [12], a number of studies have focused on this class of GSTs in human and rat tissues [13,14]. Although mouse is a model frequently used for toxicological research, very few studies on mouse GSTs have been done so far. We [15] and others [16,17] have demonstrated the selective induction of specific GST isoenzymes in mouse lung [15] and liver [16,17] in response to dietary antioxidants such as *t*-butylated hydroxyanisole (BHA). This has been proposed as a mechanism by which antioxidants mediate retardation of chemical carcinogenesis. To evaluate the significance of GST isoenzymes in mouse, we have recently studied their expression in various mouse tissues [18]. Our results demonstrated differential ex-

pression of Alpha, Mu and Pi class GSTs in the various tissues investigated [18]. We have also demonstrated the expression in mouse lung of an isoenzyme that does not appear to correspond to any of the three known major classes of GSTs [19]. In the present studies we have characterized the structural, immunological and kinetic properties of this mouse lung isoenzyme, which has a pI value of 5.7. The expression of this isoenzyme has also been demonstrated in mouse liver. Our results indicate that GST 5.7 is indeed a novel mouse GST, but is structurally similar to rat GST 8–8, which has previously been classified into the Alpha class [20] on the basis of its primary structure.

MATERIALS AND METHODS

Animals

Female CD-1 mice (virus-antibody-free), approx. 6–8 weeks of age, were purchased from Charles River Laboratories, Wilmington, MA, U.S.A., and were housed at the University of Texas Medical Branch Animal Care Center. Animals were killed by cervical dislocation, and lung and liver tissues were excised, washed once with phosphate-buffered saline (PBS; 10 mM-potassium phosphate buffer, pH 7.0, containing 150 mM-NaCl) and stored at –20 °C until used for isolation and characterization of GST isoenzymes.

Materials

Reagents for SDS/PAGE and transblotting were obtained from Bio-Rad, Richmond, CA. Epoxy-activated Sepharose 6B, CNBr-activated Sepharose 4B, staphylococcal Protein A, per-

Abbreviations used: GST, glutathione *S*-transferase; BHA, *t*-butylated hydroxyanisole; PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride); CDNB, 1-chloro-2,4-dinitrobenzene; BSP, bromosulphophthalein; *I*₅₀, concentration of inhibitor required to decrease enzyme activity by 50 %.

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oxidase-conjugated goat anti-rabbit IgG, and CNBr were procured from Sigma Chemical Company, St. Louis, MO, U.S.A. Immobilon poly(vinylidene difluoride) (PVDF) transfer membranes were from Millipore, Bedford, MA, U.S.A. Sources of all other chemicals were the same as those reported by us previously [16,20].

Purification protocol

Purification of GST isoenzymes was carried out in a manner similar to that described by us previously [21]. Briefly, a 10% (w/v) homogenate of the washed lung or liver tissue was prepared in 10 mM-potassium phosphate buffer, pH 7, containing 1.4 mM- β -mercaptoethanol (buffer A), centrifuged, dialysed, and subjected to GSH affinity chromatography by the method of Simons & Vander Jagt [22], using GSH coupled to epoxy-activated Sepharose 6B. The enzyme was eluted with 50 mM-Tris/HCl, pH 9.6, containing 1.4 mM- β -mercaptoethanol and 10 mM-GSH. Individual GST isoenzymes were separated by column isoelectric focusing as described previously [21]. All the above procedures were carried out at 4 °C. Eluted fractions were monitored for pH and GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate.

Immunoaffinity chromatography

Immunoaffinity matrix for the separation of Alpha, Mu and Pi class GSTs was prepared by coupling antibodies specific for each of these classes to CNBr activated Sepharose 4B by the method of Porath *et al.* [23]. For separation of individual classes of purified GST isoenzymes from contaminating isoenzymes of another class, the isoenzyme purified by isoelectric focusing was incubated with the antibody-coupled resin for 4 h at 4 °C, and the unabsorbed enzyme was separated by centrifugation at 1300 *g* for 15 min. The resin was then washed thoroughly with coupling buffer (0.1 M-NaHCO₃/0.5 M-NaCl, pH 7.9) and the bound enzyme was eluted with 4 M-KCNS and immediately subjected to dialysis against buffer A to remove KCNS. The unabsorbed and eluted fractions were checked for purity by Western-blot analysis.

SDS/PAGE

This was performed essentially as described by Laemmli [24], in a Bio-Rad mini-electrophoresis cell. In gels used for the determination of subunit molecular mass, 5 cm of a 12.5% resolving gel was layered with 2.5 cm of 7.1% stacking gel. For analysis of CNBr-digested peptides, a two-step gradient, consisting of 2.5 cm of 15% acrylamide overlaid with 2.5 cm of 12.5% acrylamide, was used as the resolving gel. Bands were revealed by silver staining (Morrissey [25]).

CNBr cleavage and peptide mapping

CNBr digestion of purified GST isoenzymes was carried out as described previously [26]. Briefly, freeze-dried GST protein was incubated with a 250-fold molar excess of CNBr in 70% formic acid and incubated in the dark for 48 h at room temperature. The digested samples were dried under a nitrogen atmosphere, solubilized in 60 mM-Tris/HCl, pH 6.8, containing 2% SDS and 0.5 M- β -mercaptoethanol, and subjected to SDS/PAGE as described above. For *N*-terminal sequencing studies of the peptides, the CNBr-digested peptides of GST 5.7 were obtained by treating 25 μ g of purified protein with a 500-fold molar excess of CNBr, and the reaction was allowed to progress for 60 h to ensure its completion.

N-Terminal amino acid sequencing

For *N*-terminal amino-acid-sequence analysis, samples were prepared by transblotting on Immobilon-P PVDF membranes

essentially by the method of Matsudaira [27]. Samples were first subjected to SDS/PAGE and transblotted on to Immobilon-P PVDF transfer membranes. The blots were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 reagent in 45% (v/v) methanol/10% (v/v) acetic acid, destained with 90% methanol and 7% acetic acid, rinsed with distilled water and dried. The stained bands were cut with a razor blade and loaded on to the sequencer for microsequence analysis, using an Applied Biosystems model 475A protein peptide sequencer equipped with an on-line model 120A microbore h.p.l.c. phenylthiohydantoin analyser and a model 900 data processor [26].

Enzyme assays

GST activities towards CDNB, ethacrynic acid, and bromosulphophthalein (BSP) as the electrophilic substrates were determined spectrophotometrically by monitoring the change in absorbance at 340 nm, 270 nm, and 330 nm respectively, by the method of Habig *et al.* [28]. GSH peroxidase activity was measured by the method of Awasthi *et al.* [29], except that 0.1 mM-cumene hydroperoxide was used instead of *t*-butylated hydroperoxide. A unit of GST activity was defined as the amount required for the conjugation of 1 μ mol of the electrophilic substrate to GSH/min at 25 °C, and 1 unit of GSH peroxidase activity was defined as the amount utilizing 1 μ mol of NADPH/min at 37 °C. Protein content was estimated by the method of Bradford [30], with BSA as the standard.

Western blotting

Transblotting of proteins separated by SDS/PAGE was done by the method of Towbin *et al.* [31], and blotted membrane filters were processed as described by Ahmad *et al.* [32], using polyclonal antibodies raised in rabbits [18] that were specific for each of the three classes of GSTs. Peroxidase-conjugated goat anti-rabbit IgG served as the secondary antibody. The blots were developed with peroxidase substrates, H₂O₂ and 4-chloro-1-naphthol.

Immunotitrations

Aliquots of purified enzyme preparations, containing equal amount of protein, were incubated at 37 °C for 60 min with increasing concentrations of appropriately diluted antibody in PBS containing 0.1% BSA. The antigen-antibody complex was precipitated by the addition of staphylococcal Protein A, and pelleted by centrifugation. The supernatant was evaluated for residual enzyme activity. Controls consisted of samples that were incubated with PBS containing 0.1% BSA alone.

Kinetic studies

GST activity was measured using various concentrations of one substrate, with fixed (1 mM) concentrations of the other, and double-reciprocal plots of 1/enzyme activity versus 1/[S] were used to determine *K_m* values for GSH and CDNB. For determination of *I₅₀* values, reaction cuvettes containing the enzyme, inhibitor and 100 mM-potassium phosphate buffer, pH 6.5, were incubated at 37 °C for 5 min before the addition of the substrates, GSH and CDNB. Enzyme activities in the presence of different concentrations of the inhibitor, haematin or BSP, but fixed concentrations of GSH (1 mM) and CDNB (1 mM), were estimated and plotted as percentages of control activity versus log [I]. The inhibitor concentration required to reduce the enzyme activity by 50% was determined from the plots. For determination of the inhibitor constant, *K_i*, enzyme activity was measured at each inhibitor concentration with various concentrations of the electrophilic substrate, CDNB, while the concentration of the nucleophilic substrate, GSH, was maintained at 1 mM. Double-reciprocal plots of 1/*v* versus 1/[S] were used to determine the nature of inhibition, and replots of *y*-intercept or

slope versus inhibitor concentration ([I]) were used to determine K_i values.

RESULTS

Total GST isoenzymes of mouse lung were purified by affinity chromatography over a column of GSH linked to epoxy-activated Sepharose 6B, with an overall yield of approx. 35%, and individual GST isoenzymes were separated by column isoelectric focusing. The GST isoenzyme profile was similar to that reported by us previously [19], and peaks having pI values of 9.9, 8.6, 7.7, 6.8, and 5.7 were obtained (results not shown). As determined previously [19], Western-blot analysis using polyclonal antibodies specific for each of the three classes of GSTs and *N*-terminal amino acid sequencing indicated that GST 9.9 belonged to the Alpha class and GST 7.7 and GST 6.8 belonged to the Mu class. The peak at pH 8.6 represented a mixture of two distinct isoenzymes, one belonging to the Pi class and the other to the Mu class, which were separated by immunoaffinity chromatography as described in the Materials and methods section. Subunits belonging to GST 5.7 had blocked *N*-termini, similar to those of Alpha class GSTs, and did not cross-react with antibodies specific for the Alpha, Mu or Pi class GSTs. These results are consistent with our previous finding [19]. The present studies were primarily focused on the characterization of mouse lung GST 5.7, but representative isoenzymes of mouse lung belonging to the Alpha (GST 9.9), Mu (GST 7.7) and Pi (GST 8.6) were also analysed for comparison with GST 5.7.

Structural characterization of GST 5.7

The Alpha (pI 9.9), Mu (pI 7.7), and Pi (pI 8.6) class GST isoenzymes and GST 5.7 of mouse lung were analysed by SDS/PAGE along with molecular-mass markers and GSH-affinity-purified total GST (Fig. 1). Whereas the molecular-mass values for the Alpha (25 kDa), Mu (26.5 kDa) and Pi (22.5 kDa) class GST subunits were the same as those reported previously [19] (Fig. 1; lanes 2, 3 and 4 respectively), GST 5.7 subunits had a mobility intermediate between that of the Alpha and that of the Pi class subunits, i.e. approx. 23 kDa (Fig. 1, lane 5).

Peptide mapping

Peptide maps of GST isoenzymes of mouse lung digested with CNBr under identical conditions were compared to determine if GST 5.7 was structurally related to any of the three known classes of GST isoenzymes. Equal amounts (2 μ g of protein) of

Fig. 2. Peptide maps of CNBr digests of mouse lung GST isoenzymes

A 2 μ g portion of immunoaffinity-purified protein of individual mouse lung GST isoenzymes was treated with CNBr as described in the Materials and methods section. Peptide fragments were separated by SDS/PAGE, and bands were revealed by silver staining. Lanes 1, 3, 5, and 7 contained 0.2 μ g of untreated GST 9.9 (Alpha class), GST 7.7 (Mu class), GST 8.6 (Pi class) and GST 5.7 respectively. Lanes 2, 4, 6, and 8 correspond to 2 μ g samples of GST 9.9, GST 7.7, GST 8.6, and GST 5.7 (respectively) treated with CNBr.

purified preparations of GST 9.9 (Alpha class), GST 7.7 (Mu class), GST 8.6 (Pi class) and GST 5.7 were incubated with a 250-fold molar excess of CNBr for 48 h at 25 °C in the dark. After the reaction, the samples were dried under nitrogen and subjected to SDS/PAGE as described in the Materials and methods section. The gel was developed by silver staining, and the results presented in Fig. 2 indicated that, apart from some intact GST subunit, each class of GST isoenzymes had a characteristic peptide map, owing to differences in the number and position of methionine residues in their primary structures. It was notable that the peptide map of GST 5.7 did not resemble those of any of the three other isoenzymes, suggesting that it was structurally distinct from the Alpha, Mu and Pi class GSTs. When GST 5.7 was treated for 60 h using a 500-fold molar excess of CNBr, only two major peptide bands with molecular-mass values of 6 kDa and 12 kDa could be detected (Fig. 3, lanes 3 and 5).

Expression of GST 5.7 in mouse liver

We have also identified a GST isoenzyme in mouse liver, with a pI value of 5.7, which did not correspond to the Alpha, Mu or Pi class GSTs. Structural, kinetic and immunological properties

Fig. 1. SDS/PAGE gel showing distinct molecular mass of GST 5.7

Lanes 2–5 represent GST isoenzymes of mouse lung corresponding to Alpha (pI 9.9), Mu (pI 7.7), and Pi (pI 8.6) classes, and GST 5.7 respectively. Lane 6 corresponds to GSH-affinity-purified total mouse lung GST, and lanes 1 and 7 contain molecular-mass markers. The gel was developed by silver staining.

Fig. 3. Comparison peptide maps of GST 5.7 of mouse lung and liver

Lanes 2 and 4 correspond to 0.2 μ g of GST 5.7 of mouse lung and liver respectively. Lanes 3 and 5 correspond to 2 μ g of GST 5.7 of mouse lung and liver respectively digested for 60 h with a 500-fold molar excess of CNBr. Lane 1 corresponds to molecular-mass markers.

Fig. 4. Alignment of N-terminal amino acid sequence of CNBr peptides

Fragments (6 and 12 kDa) of mouse lung and liver GST 5.7 were obtained by CNBr digestion. CNBr cleavage was done by incubating 25 µg of purified GST 5.7 with a 500-fold molar excess of CNBr for 60 h in the dark. The peptide fragments were separated by SDS/PAGE, trans-blotted, and the bands cut off and subjected to N-terminal amino acid sequencing. The positions of the sequences of rat GST 8-8 that align with the sequences of the CNBr peptides of GST 5.7 have been indicated by superscripts on the first and last residues of the sequences. After alignment, those residues that are different have been boxed. Sources of sequence information: * Alin *et al* [20]; † the present study.

Table 1. Specific activities of mouse lung GST isoenzymes

All values represent averages of three determinations. Abbreviation: N.D., not detected.

GST	Class	Substrate...	GST specific activity (µmol of conjugate formed/min per mg of protein at 25 °C)			GSH peroxidase specific activity (µmol of NADPH utilized/min per mg of protein at 37 °C)
			CDNB	Ethacrynic acid	BSP	
9.9	Alpha		3.41	0.17	N.D.	11.50
8.6	Pi		29.76	1.98	0.13	N.D.
7.7	Mu		21.32	0.68	0.44	N.D.
5.7			21.93	1.82	0.10	N.D.

of the liver enzyme showed close similarity with those of GST 5.7 of mouse lung. Western-blot analysis of liver isoenzyme, using polyclonal antibodies specific for each of the three major known classes of GSTs, also failed to show any cross-reactivity (results not shown). In SDS-containing gels, GST 5.7 of mouse liver (Fig. 3, lane 4) co-migrated with GST 5.7 of mouse lung (Fig. 3, lane 2), and had a subunit size of 23 kDa. The liver enzyme also failed to yield an N-terminal amino acid sequence, indicating a blocked N-terminus, similar to that of GST 5.7 of mouse lung. CNBr digestion of mouse liver and lung GST 5.7 run simultaneously for 60 h using a 500-fold molar excess of CNBr showed identical peptide maps for both enzymes, with two major peptides having sizes of about 12 and 6 kDa in each case (Fig. 3, lanes 3 and 5). Similarly, incomplete digestion of liver GST 5.7 with a 250-fold molar excess of CNBr for 48 h resulted in a peptide profile similar to that obtained for the lung enzymes under identical

Fig. 5. Western-blot analysis of purified GST 5.7 of mouse lung

Approx. 2 µg protein of purified GST isoenzymes were subjected to Western blotting. Blots were developed using polyclonal antibodies raised against the Alpha class GSTs (α-ε group) from human liver (a), the Mu class GST (GST ψ) of human liver (panel B) and GST π of human placenta (c). Lane 1 in each panel is the positive control against which the respective antibodies were raised. Lane 2 corresponds to mouse lung Alpha class GST 9.9 (a), Mu class GST 7.7 (b), and Pi class GST 8.6 (c). Lanes 3 and 4 correspond to GST 5.7 and affinity-purified total GST of mouse lung respectively.

conditions (results not shown). Substrate reactivities, kinetic properties and inhibition characteristics of the liver enzyme (results not shown) were also similar to the lung enzyme GST 5.7, suggesting that the two were related, and probably identical, proteins.

***N*-Terminal sequencing and alignment of CNBr digestion products**

Since the *N*-terminus of GST 5.7 was blocked, a direct comparison of the *N*-terminal primary structure with Alpha, Mu and Pi class GST subunits was not possible. Therefore, lung and liver GST 5.7 (25 µg each) were separately treated with a 500-fold molar excess of CNBr for 60 h in dark, and the resulting peptides were separated by SDS/PAGE, and transblotted on to Immobilon-P PVDF transfer membranes for *N*-terminal amino acid sequencing. The peptide bands were revealed by staining with Coomassie Blue R, and two major bands, corresponding to approx. 12 kDa and 6 kDa, were detected. A sequence of up to 15 residues was obtained for the 6 kDa band generated from lung GST 5.7. This sequence was compared with known sequences of various mammalian cytosolic and microsomal GSTs and was found to match the sequence of rat GST 8–8 at positions 106–120 with a 73% identity (Fig. 4a). This suggested that GST 5.7 was the mouse equivalent of rat GST 8–8 which has been previously designated as an Alpha class isoenzyme on the basis of its primary structure [20]. The 12 kDa CNBr fragment of lung GST 5.7 did not transfer efficiently to the Immobilon membrane, resulting in a low yield that was insufficient to generate a sequence. Since we had identified this isoenzyme from mouse liver and it was possible to obtain higher yields from liver, further structural analysis of GST 5.7 was done after its purification from mouse liver. 6 kDa and 12 kDa peptides were generated by CNBr digestion of liver GST 5.7, using the conditions described in the Materials and methods section, transferred to Immobilon-P PVDF transfer membranes after SDS/PAGE and subjected to

N-terminal amino acid sequencing. The 6 kDa band from mouse liver enzyme generated two sequences. The eight-residue minor sequence was identical with that of the 6 kDa fragment of mouse lung GST 5.7 (Fig. 4a). In addition, another sequence of 20 residues was also obtained that matched rat GST 8–8 at positions 167–186 with a 79% identity (Fig. 4b), further suggesting that mouse GST 5.7 might be homologous with rat GST 8–8. The 12 kDa fragment gave a sequence identical with the minor sequence generated by the 6 kDa fragment, suggesting that it probably was a product of incomplete digestion. Our peptide-fragmentation pattern and sequencing data suggest that two 6 kDa peptides were generated that corresponded to fragments CN28 and CN23 reported by Alin *et al.* [20], and spanned residues 106–165 and 167–222 respectively of GST 8–8. These failed to be resolved on SDS/PAGE because of very similar molecular-mass values. Incomplete cleavage of these peptides resulted in the 12 kDa fragment representing residues from 106 to the C-terminus. These results also confirm that GST 5.7 isolated from mouse liver and lung are identical proteins.

Reactivity of GST 5.7 towards preferred substrates for the Alpha, Mu and Pi classes

Specific activities of Alpha, Mu and Pi class GSTs towards various substrates were compared with those of GST 5.7, and the data are presented in Table 1. Unlike the Alpha class isoenzyme, GST 5.7 did not exhibit GSH peroxidase activity towards cumene hydroperoxide. On the contrary, its activity towards ethacrynic acid was comparable with that of the Pi class isoenzyme GST 8.6 of mouse lung. The specific activity of GST 5.7 towards CDNB was also much higher than that of GST 9.9 (Alpha class), and similar to, although not as high as, that of the Pi class GST 8.6. The substrate reactivities of the Mu class GST 7.7 were also distinct from those of GST 5.7.

Immunological characterization

The immunological relationship of GST 5.7 to Alpha, Mu and Pi class GSTs was evaluated by Western-blot analysis, using antibodies specific for each of the three classes of GSTs. As demonstrated in Fig. 5, GST 5.7 (lane 3, in *a*, *b* and *c*) did not cross-react with anti-Alpha (*a*), anti-Mu (*b*) or anti-Pi (*c*) class antibodies, indicating that it is an immunologically distinct protein. Lanes 1 and 2 in Figs. 5(*a*), 5(*b*) and 5(*c*) represent GST isoenzymes of human tissues and mouse lung belonging to the corresponding classes respectively and showed a positive reaction, as expected. The immunological relationship of GST 5.7 to the Alpha and Mu classes of GST was further investigated in immunoprecipitation experiments using polyclonal antibodies specific for the Alpha and the Mu class GSTs. Whereas the Alpha and Mu class antibodies did precipitate GST 9.9 and GST 8.6 respectively, GST 5.7 was not precipitated with either of the antibodies, as evidenced by retention of most of the GST activity

Table 2. K_m values of mouse lung GST isoenzymes for GSH and CDNB

K_m values were calculated from double-reciprocal plots of $1/v$ versus $1/[S]$. The values represent means of three similar values.

GST	Class	K_m (mM)	
		GSH	CDNB
9.9	Alpha	0.30	2.30
8.6	Pi	0.84	0.83
7.7	Mu	0.48	0.38
5.7		4.00	0.80

Table 3. Comparison of the kinetics of inhibition of mouse lung GST isoenzymes

K_i values were calculated from the double-reciprocal plots shown in Figs. 6(*a*) and 6(*b*). Abbreviation: N.D., not determined.

GST	Class	Haematin			BSP		
		I_{50} (µM)	K_i (µM)	Nature of inhibition	I_{50} (µM)	K_i (µM)	Nature of inhibition
9.9	Alpha	0.09	0.43	Competitive	35.50	8.28	Non-competitive
8.6	Pi	0.08	0.05	Non-competitive	8.91	11.68	Competitive
7.7	Mu	1.26	N.D.	N.D.	59.56	N.D.	N.D.
5.7		0.11	0.21	Non-competitive	1.26	5.30	Uncompetitive

Fig. 6. Determination of K_i values of haematin (a) and BSP (b) for mouse lung GSTs

GST activities of Alpha class GST 9.9 (panel A₁), Pi class GST 8.6 (panel B₁) and GST 5.7 (panel C₁) were determined at fixed concentrations of GSH (1 mM) and variable concentrations of CDNB (0.25–2.00 mM) in the absence (○) or presence of 0.05 (●) or 0.50 (0.1 μ M for Pi class GST 8.6; panel B₁) (Δ) μ M-haematin (a) or 1 (●) or 10 (Δ) μ M-BSP (b). Double-reciprocal plots were used to determine the nature of inhibition. Each point was a mean result of three determinations. Panels A₂, B₂ and C₂ represent replots of y-intercept or slope against inhibitor concentration. The x-intercept represents $-K_i$.

in the supernatant (results not shown). These data, taken together with the results of Western blotting, indicate that GST 5.7 is immunologically distinct from Alpha Mu and Pi class GSTs.

Kinetic and inhibition studies

The kinetic and inhibition properties of GST 5.7 were also compared with those of Alpha, Mu and Pi class isoenzymes of

mouse lung, to determine its functional relationship to other known GST isoenzymes. The K_m values for GSH and CDNB for each of the isoenzymes were variable (Table 2), but within the range observed for other rat and human isoenzymes studied [33]. GST 5.7 had a K_m for GSH of 4 mM, which was higher than reported for most other GSTs. I_{50} and K_i values, and the nature of inhibition of two characteristic inhibitors, haematin and BSP, for the various isoenzymes of mouse lung were determined to evaluate the relationship of GST 5.7 with the Alpha, Mu and Pi classes (Table 3; Figs. 6a and 6b). These data indicated that GST 5.7 could not, on the basis of kinetic properties, be identified with any one of the Alpha, Mu or Pi isoenzymes studied.

DISCUSSION

The most intriguing feature of GSTs is their tissue specificity, multiplicity of expression and microheterogeneity. Several studies over the past decade have revealed the existence of multiple genes encoding the various GST subunits known so far [3,9–11]. Though a majority of the cytosolic GSTs fall into three distinct categories, Alpha, Mu and Pi [3,4], the existence of additional class(es) or subunits is not ruled out from structural studies [34,35], and starch-gel-electrophoretic analysis [36]. Present studies were aimed at characterizing a novel isoenzyme from mouse lung and liver. We have provided evidence that this isoenzyme, mouse GST 5.7, has characteristic structural, kinetic and immunological properties that enable its identification as the mouse orthologue of rat GST 8–8. The apparent subunit size of mouse GST 5.7 is, however, smaller than that reported for rat GST 8–8 [20]. Rat GST 8–8 has been classified previously as belonging to the Alpha class on the basis of its primary structure [20,37], despite having significant differences in its kinetic and immunological properties as compared with other mammalian Alpha class isoenzymes, such as rat GST 1–1, GST 2–2, human Ha₁, Ha₂, Skin 9.9 and mouse GST MI [3]. Present studies indicate that mouse GST 5.7 is immunologically distinct from Alpha as well as Mu and Pi class GSTs and has a characteristic antigenic nature, both in the native and in the denatured state. Rat GST 8–8 has also been shown to be immunologically distinct from Alpha, Mu and Pi classes. Taken together, these findings may indicate that rat GST 8–8 and mouse GST 5.7 have a relatively more distant evolutionary relatedness to the other Alpha class isoenzymes.

Substrate specificities of mouse GST 5.7 are also significantly different from other Alpha class GSTs. Unlike most Alpha class isoenzymes, it exhibited high activity towards ethacrynic acid, a substrate known to be a preferred substrate for Pi class isoenzymes. This has also been reported for rat GST 8–8 [20]. However, unlike rat GST 8–8, mouse GST 5.7 did not exhibit any activity towards cumene hydroperoxide, which is a known preferred substrate for the Alpha class isoenzymes. These data suggest that the substrate reactivities of GST 5.7 are closer to the substrate preferences reported for the Pi class GST rather than Alpha class GSTs. It is noteworthy that, similarly to mouse GST 5.7, rat GST 8–8 [20], human skin GST '9.9' [38], as well as the Alpha class anionic isoenzyme characterized from human liver [39], also have relatively higher specific activities towards ethacrynic acid. Some of the recent studies suggest that several Alpha class GST isoenzymes show rather large differences among themselves in regards to their substrate specificities. For example, unlike the generally accepted substrate preferences of the Alpha class GST isoenzymes, mouse GST 5.7 characterized in the present study has no detectable GSH peroxidase activity, rat GST 8–8 has relatively very high activity towards ethacrynic acid [20] and the anionic Alpha class GST of human liver expresses GSH peroxidase activity towards H₂O₂ [39]. It is possible that the

Alpha class mammalian GSTs may actually be a more heterogenous group than currently realized, and the isoenzymes within this group have considerable functional and immunological dissimilarities. Further studies are needed to elucidate structural, functional and kinetic interrelationships among the Alpha class mammalian GSTs.

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