Glutathione analogue sorbents selectively bind glutathione S-transferase isoenzymes

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Novel affinity sorbents for glutathione S-transferases (GSTs) were created by binding glutathione (GSH) analogues to Sepharose 6B. The GSH molecule was modified at the glycine moiety and at the group attached to the sulphur of cysteine. When tested by affinity chromatography in a flow-through microplate format, several of these sorbents selectively bound GST isoenzymes. γ E-C(Hx)- ϕ G (glutathione with a hexyl moiety bound to cysteine and phenylglycine substituted for glycine)

specifically bound rat GST 7-7, the Pi-class isoenzyme, from liver, kidney and small intestine. γ E-C(Bz)- β A (benzyl bound to cysteine and β -alanine substituted for glycine) was highly selective for rat subunits 3 and 4, which are Mu-class isoenzymes. By allowing purification of the isoenzymes under mild conditions that preserve activity, the novel sorbents should be useful in characterizing the biological roles of GSTs in both normal animal and cancer tissues.

INTRODUCTION

The glutathione S-transferases (GSTs; EC 2.5.1.18) are a group of multifunctional, primarily cytosolic, enzymes that catalyse the nucleophilic attack of glutathione (GSH) on electrophilic centres in a wide variety of endogenous and exogenous organic molecules. In addition to the soluble isoenzymes, distantly related microsomal membrane-bound forms have been identified (Morgenstern and DePierre, 1988; Söderström et al., 1988), but these will not be considered in this paper. The soluble GST isoenzymes exhibit broad, overlapping substrate specificities and may also function as significant ligand binding proteins, given their high abundance, roughly 1 % of total cell protein (Listowsky et al., 1988; Mannervik and Danielson, 1988; Ketterer et al., 1988). The isoenzymes have been grouped into four species-independent classes: Alpha, Mu, Pi and Theta, based on their substrate and inhibitor binding properties, their immunological cross-reactivities and the similarity of their primary structures, as determined by analysis of recombinant DNA (Mannervik et al., 1985, 1992; Meyer et al., 1991). The enzymes include both homo- and hetero-dimers formed from subunits within a single class, with a subunit M_r ranging from 23000 to 28000. GSTs show unique patterns of tissue-specific distribution in both rodents and humans (Mannervik and Danielson, 1988; Ketterer et al., 1988).

GSTs appear to play a major role in the multifactorial process of drug resistance in tumours. Because alkylating agents contain strong electrophilic centres, they have a high potential for forming stable conjugates with GSH. Melphalan (Dulik et al., 1986), chlorambucil (Dulik et al., 1990), cyclophosphamide and phosphoramidate mustard (McGown and Fox, 1986), the cyclophosphamide metabolite acrolein (Berhane and Mannervik, 1990) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) (Smith et al., 1989) all form GSH–drug conjugates in reactions catalysed by the GSTs. Analysis of human tumour specimens and cell lines shows that the Pi-class isoenzyme is expressed at high concentrations in the majority of such samples (Moscow et al., 1989;

Howie et al., 1990; Castro et al., 1990). Furthermore, increased expression of specific GST isoenzymes in tumour cell lines has been associated with the acquired resistant phenotype to chemotherapeutic drugs (Batist et al., 1986; Buller et al., 1987; Robson et al., 1987; Smith et al., 1989; see Wolf et al., 1990, for a review). Direct evidence for the role of elevated GST activities in mediating anti-tumour drug resistance was provided by the demonstration that transient expression of human and rat GST genes in cultured COS monkey kidney cells resulted in moderate but clinically significant resistance to several alkylating agents (Puchalski and Fahl, 1990). Treatment with relatively non-toxic GST inhibitors, such as triphenyltin chloride, ethacrynic acid and piriprost, results in significant sensitization of cultured cells to alkylating agents (Tew et al., 1988; Smith et al., 1989; Clapper et al., 1989; Hansson et al., 1991). In addition, *in vitro* and preclinical studies have demonstrated the chemosensitizing effect of reduced cellular GSH pools via the drug buthionine sulfoximine (BSO), an inhibitor of *de novo* GSH synthesis (Meister, 1988).

The cytosolic isoenzymes from rat are the best understood in terms of structure, functions and tissue distribution (Ketterer et al., 1988). Of particular relevance to the present work, enzymological tests on individual rat isoenzymes using a variety of GSH analogues has led to a general model for the GSH-binding site, or G-site, of the GSTs (Adang et al., 1990). Studies by other investigators have defined an additional H-site at which moderately hydrophobic substrates bind (Mannervik and Danielson, 1988).

Typically, the initial step in the characterization of cytosolic GSTs involves affinity chromatography on S-linked GSH–Sepharose (S-L-GSH) or S-hexylglutathione (HxGSH), which bind all GST isoenzymes. This step is relatively simple. If characterization of specific isoenzymes is needed, the elution with free affinity ligand must be followed by additional procedures, such as chromatofocusing, ion-exchange chromatography, SDS/PAGE or reverse-phase h.p.l.c. (RP-

Abbreviations used: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CDNB, 1-chloro-2,4-dinitrobenzene; CF, compensatory factor; DTT, dithiothreitol; GST, glutathione S-transferase; HxGSH, S-hexylglutathione; PMSF, phenylmethanesulphonyl fluoride; RP-h.p.l.c., reverse-phase h.p.l.c.; S-L-GSH, S-linked glutathione–Sepharose; TFA, trifluoroacetic acid.

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Table 1 Characteristics of glutathione analogue substituents

Abbreviations used: G, glycine; A, alanine; β A, β -alanine; ϕ G, phenylglycine; H, histidine; D, aspartate; V, valine; Bz, benzyl; BuBz, 4-tert-butylbenzyl; Cu, cumyl (= α , α -dimethylbenzyl); Hx, hexyl; Pr, propyl.

Terminal amino acid (Y)	Substituent on the S of cysteine (X)					
	Hydrogen	Propyl	Hexyl	Benzyl	Cumyl	BuBz
G	γ E-C(H)-G		γ E-C(Hx)-G		γ E-C(Cu)-G	γ E-C(BuBz)-G
A		γ E-C(Pr)-A		γ E-C(Bz)-A		
β A				γ E-C(Bz)- β A		
ϕ G			γ E-C(Hx)- ϕ G	γ E-C(Bz)- ϕ G		
H		γ E-C(Pr)-H		γ E-C(Bz)-H		
D		γ E-C(Pr)-D	γ E-C(Hx)-D			
V		γ E-C(Pr)-V	γ E-C(Hx)-V			

h.p.l.c.). These procedures are laborious and often require large amounts of materials, which are frequently not available. An additional disadvantage of the latter two procedures is that they result in denaturation of the isoenzymes, precluding subsequent studies on the activities of the isolated isoenzymes.

In this paper, we demonstrate that systematic concurrent modification of GSH at the C-terminal glycine residue and at the S-linked group on cysteine produces specific affinity ligands capable of purifying individual rat cytosolic GSTs which appear to retain fully their native structure and activity. A rapid, small-scale method for the affinity separation of the GSTs is also described, extending prior work with other families of diversified, multi-mode chromatography ligands (paralogues) (Benedek et al., 1992).

MATERIALS AND METHODS

Chemicals

Epoxy-activated Sepharose 6B was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). GSH (Sigma, St. Louis, MO, U.S.A.) was coupled to the Sepharose 6B through the SH-group on cysteine (S-linked GSH) by the method of Simons and Vander Jagt (1977). HxGSH was synthesized by the method of Vince et al. (1971). The novel GSH analogues in this study were synthesized at Terrapin Technologies, Inc. (South San Francisco, CA, U.S.A.) by adapting the general *N*-fluorenylmethoxycarbonyl (Fmoc)-mediated solid-phase peptide synthesis method (Carpino and Han, 1972) to liquid-phase synthesis (Lytle et al., 1992). HxGSH and the novel GSH analogues were coupled to the Sepharose 6B via the free NH_2 group on glutamic acid in 33% ethanol adjusted to pH 11 (Lytle et al., 1992). Silent Monitor 96-well membrane-bottomed microplates were purchased from Pall Biosupport (East Hills, NY, U.S.A.). To prevent proteins sticking to the plastic, standard microplates (Costar, CA, U.S.A.) were routinely soaked in 0.1% Tween, rinsed and dried before use. Protein Assay Dye Reagent Concentrate was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were reagent grade commercial products.

Sorbent nomenclature

Systematic sampling of GSH analogues varying concurrently in properties believed to be relevant to binding to both G- and H-sites on GSTs yielded a manageable number of sorbents that could be synthesized and tested for their abilities to bind GST isoenzymes. A selection of the 75 sorbents developed and tested at Terrapin is listed in Table 1. The system for naming these sorbents, which extends the standard single letter abbreviations for the amino acids, begins with the γ -glutamate (γ E) followed by the cysteine. The moiety bound to the sulphur of cysteine is listed in parentheses. The final amino acid is listed last. By this convention, γ E-C(Hx)- ϕ G represents an analogue with an S-linked hexyl group and phenylglycine substituted for the glycine.

Preparation of crude cytosolic fractions

Untreated female Sprague-Dawley rats were anaesthetized with CO_2 and killed by exsanguination. Liver, lung and kidney were immediately removed, rinsed in ice-cold saline and homogenized in 10 mM KH_2PO_4 , pH 7.0, 150 mM KCl, 2 mM dithiothreitol (DTT) and freshly prepared 250 μM phenylmethanesulphonyl fluoride (PMSF). Cytosol was prepared by ultracentrifugation at 105000 *g* for 1 h at 4 °C. The first 30 cm of small intestine was removed, rinsed with ice-cold 150 mM KCl containing 250 μM PMSF, and slit open, and the mucosa was removed by three gentle scrapings along the entire length. The mucosa was homogenized and cytosol prepared as for liver.

Affinity chromatography

Affinity chromatography was performed in 96-well microplates having a bottom surface of a 1.2 μm Loprodyn membrane. The use of these microplates in chromatography has been described earlier (Kauvar et al., 1990). Briefly, 400 μl of a 50% slurry of each of the sorbents was pipetted into appropriate wells in the membrane-bottomed microplate, and the resulting micro-affinity columns (200 μl settled bed volume) were equilibrated with TE buffer (10 mM Tris/HCl, pH 7.8 at 20 °C, 1 mM EDTA, 1 mM DTT and 100 μM PMSF) by two cycles of centrifugation at 650 *g* for 30 s. A 2 mg sample of cytosol (~75 μl of crude liver

cytosol) was applied to the semi-dry sorbents and incubated with continuous shaking in a Minimix vibrator (Fisher, CA, U.S.A.) for 1 h at 4 °C. The sorbent microplate was placed above a recipient standard Tween-treated microplate and centrifuged to recover the flow-through fractions. To ensure full adsorption, the flow-through material was re-applied to the sorbents for a 10 min re-incubation and centrifuged again. The sorbents were washed by several cycles of centrifugation with $4 \times 150 \mu\text{l}$ of TE buffer and $6 \times 150 \mu\text{l}$ of TEN buffer (TE buffer containing 200 mM NaCl). The retained proteins were eluted in three fractions of $150 \mu\text{l}$ each with 10 mM HxGSH in TEN buffer. Following conditions used elsewhere for GST separation (Mannervik et al., 1985; Meyer et al., 1989), HxGSH was used as the eluant for this new procedure. A high concentration, 10 mM, was needed for maximum protein yield from the micro-columns. Elution at pH 7.8 and pH 9.6 produced similar yields, but the isoenzymes were more stable at the lower pH. GSH as an eluant at 10 mM did not produce yields comparable to those with 10 mM HxGSH. Repeated analysis by RP-h.p.l.c. has shown that this HxGSH concentration generates a similar isoenzyme composition in each of the sequential eluate fractions, although at much lower levels in the later fractions; for this study the first eluate fraction was used for SDS/PAGE and RP-h.p.l.c. analysis.

Assays

GST activity was determined at 30 °C in a Beckman DU-64 spectrophotometer using a Soft-Pac Kinetics Module (Beckman Instruments, Fullerton, CA, U.S.A.) by the procedure of Habig and Jakoby (1981). Briefly, 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and 2 mM GSH were used as substrates, and changes in absorbance were monitored at 340 nm, reflecting a spectral shift in the GSH-conjugated form of CDNB. Protein concentration was determined by adapting the Bio-Rad microassay to a 96-well microplate using a Thermo-max microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.) for quantification.

Correction of GST activities towards CDNB for the inhibition caused by the HxGSH eluant

Our affinity chromatography procedures use 10 mM HxGSH as eluant, so activity measurements reflect both enzyme recovery and inhibition by HxGSH. To avoid further manipulation of the eluted isoenzymes (dilution by dialysis followed by concentration etc.), we compensated for this inhibition by using a factor calculated from the following experiment. Bulk rat liver cytosolic GST isoenzymes were affinity-purified as described above and the first two elution fractions, containing the highest protein concentration, were pooled as a source of calibration material for an inhibitor dose-response study. To remove HxGSH, the pooled fractions were extensively dialysed against TE buffer. The enzyme concentration giving maximal activity towards CDNB was determined to be $0.3 \mu\text{g/ml}$, and this was used for the calibration study. An aliquot of the calibration enzyme pool was incubated for 3 min with HxGSH. The final concentration of HxGSH in the assay mixture ranged from $0.1 \mu\text{M}$ to 1 mM, yielding 0–90% inhibition in an optimal CDNB assay. The resulting inhibition curve was well fitted by a second degree equation yielding a compensatory factor (CF) for use in correcting measured activities of eluates ($\text{CF} = -13.8x^2 + 27.7x + 1.1$; $x = \text{HxGSH concentration in mM}$).

For the experiments described in this report, the enzyme concentration in the eluates was brought to approx. $0.3 \mu\text{g/ml}$,

activities with CDNB were determined, and the raw values were multiplied by the CF for the level of HxGSH in the assay mixture. This correction is only qualitative, however. Since different GST isoenzymes show different specificities for HxGSH, a CF based on the inhibition of bulk GST activities present in rat liver cytosol is not able to correct activities of subsets of the isoenzymes with high precision. Nevertheless, since HxGSH has reasonably uniform potency as an inhibitor for all GSTs, the CF is qualitatively accurate.

Gel electrophoresis

SDS/PAGE was performed according to standard methods (Laemmli, 1970) at a 12.5% (w/v) acrylamide concentration. Proteins were visualized after electrophoresis by silver staining (Blum et al., 1987).

Analysis of the eluate composition by RP-h.p.l.c.

RP-h.p.l.c. analysis of eluates was performed by the method of Kelley and Bjeldanes (1991). A $25 \mu\text{l}$ sample, containing approx. $3 \mu\text{g}$ of protein, was separated on a $0.46 \text{ cm} \times 25 \text{ cm}$ Dynamax 300 Å C-4 reverse-phase column (Rainin Instruments, Woburn, MA, U.S.A.) using acetonitrile with 0.1% trifluoroacetic acid (TFA) and water with 0.1% TFA as solvents. A gradient from 43 to 55% acetonitrile over 38 min was used. The elution was monitored at 214 nm.

GST isoenzymes were identified by the co-elution of purified isoenzymes 1-1, 2-2, 3-3 and 3-4 (provided by Professor B. Mannervik, Department of Biochemistry, University of Uppsala, Sweden) added to the experimental sample, by comparison of retention times, and by comparison of SDS/PAGE mobilities of components of the elution fraction with literature values.

RESULTS

The recoveries of protein and CDNB-conjugating activity in the elution fractions after affinity purification of rat liver cytosolic GST isoenzymes are shown in Table 2. S-L-GSH and HxGSH sorbents showed high binding and broad isoenzyme recognition.

Table 2 Recovery of protein and CDNB-conjugating activity after affinity chromatography of rat liver cytosol on GSH and selected GSH analogue sorbents

Values are means of duplicates. A sample of 2 mg of cytosolic protein, corresponding to an activity of $1.98 \mu\text{mol/min per ml}$, was applied to each sorbent. The nomenclature and coupling of GSH and GSH analogues to epoxy-activated Sepharose 6B was as described in the Materials and methods section. Activities have been corrected for HxGSH inhibition, as described in the Materials and methods section.

Sorbent	Elution fractions			
	Protein		Activity	
	(μg)	(% of total protein)	($\mu\text{mol/min per ml}$)	(% of total activity)
HxGSH	60	3.0	1.32	67
S-L-GSH	51	2.5	1.21	61
$\gamma\text{E-C(Cu)-G}$	49	2.5	0.64	32
$\gamma\text{E-C(Bz)-}\beta\text{A}$	20	1.0	0.69	35
$\gamma\text{E-C(Bz)-A}$	11	0.5	0.43	22
$\gamma\text{E-C(Hx)-}\phi\text{G}$	4	0.2	0.10	5

Figure 1 SDS/PAGE of rat cytosol and affinity fractions

GST enzymes from rat liver and kidney were analysed by SDS/PAGE. The first three lanes analysed were: lane 1, rat liver cytosol; 2, flow-through of liver cytosol from HxGSH-Sepharose; 3, salt wash of liver cytosol from HxGSH-Sepharose. The remaining lanes are elution 1 fractions of liver and kidney cytosol from various sorbents: 4, liver HxGSH; 5, S-L-GSH; 6, γ E-C(Cu)-G; 7, γ E-C(Bz)- β A; 8, γ E-C(Bz)-A; 9, kidney γ E-C(Hx)- ϕ G. The 12.5% (w/v) polyacrylamide/0.3% (w/v) *NN'*-methylenebisacrylamide gels were loaded with 250 μ g of protein/lane for cytosol and flow-through, and with 200 ng of protein/lane for the remaining samples.

The novel sorbents retained a lower mass of the GST isoenzymes, with the protein recovery for three of the novel sorbents being less than half that of the traditional sorbents.

In comparison with HxGSH, the novel sorbents did not show a direct correspondence of CDNB-conjugating activity with protein recovery. For example, γ E-C(Bz)- β A and γ E-C(Bz)-A sorbents gave 33% and 16% of the protein recovery seen with HxGSH, but the CDNB-conjugating activities were far higher (52% and 33% respectively) after correcting for the inhibitory properties of the eluting ligand. In contrast, protein recovery by γ E-C(Cu)-G sorbent decreased minimally, while activity decreased by 50%. It is well known that different GST isoenzymes display individual specific activities with CDNB (Mannervik and Danielson, 1988). The discrepancy between protein recovery and activity recovery was attributed to differences in the isoenzyme composition in the elution fractions of each sorbent. This hypothesis was supported by SDS/PAGE (Figure 1) and RP-h.p.l.c. (Figure 2) analysis of the eluate fractions.

Fractions eluted from the sorbents listed in Table 2 were analysed by SDS/PAGE followed by silver staining (Figure 1). S-L-GSH and HxGSH sorbents bound proteins which corresponded to the previously observed electrophoretic band pattern of rat liver GST subunits (Ketterer et al., 1988). The novel sorbents displayed different band patterns. For example, the γ E-C(Bz)- β A eluate referred to above showed a highly enriched band for presumptive Mu-class isoenzymes. As the Pi-class GST 7-7 is found at low levels in rat liver cytosol, we also tested other rat tissues known to express higher levels of this isoenzyme. Figure 1 also shows the GST gel pattern from rat kidney cytosolic proteins eluted from the γ E-C(Hx)- ϕ G sorbent. A protein with the same molecular migration as rat GST subunit

Figure 2 RP-h.p.l.c. analysis of rat liver GSTs affinity-purified on HxGSH and novel sorbents

Aliquots of 25 μ l, containing 0.5–3.0 μ g, of the elution 1 fraction from each sorbent, were analysed for GST isoenzyme content by RP-h.p.l.c. on a Rainin C-4 0.45 cm $\times$ 25 cm column using acetonitrile with 0.1% (w/v) TFA and water with 0.1% TFA as solvents. A gradient of 43–55% acetonitrile over 38 min was used for the separation of the isoenzymes. The elution was monitored at 214 nm. The sorbents shown are (a) HxGSH, (b) γ E-C(Cu)-G and (c) γ E-C(Bz)- β A. The numbers above the peaks refer to rat GST subunit number.

7 is the unique component of this eluate, indicating that this sorbent is a Pi-selective reagent.

A protein band with a molecular migration similar to that of another GSH-dependent enzyme, glyoxylase I, was also present

Figure 3 RP-h.p.l.c. analysis of rat liver and kidney GSTs affinity-purified on HxGSH and γ E-C(Hx)- ϕ G

Elution fractions of liver and kidney from HxGSH and γ E-C(Hx)- ϕ G were compared to show the selectivity of the γ E-C(Hx)- ϕ G for GST 7-7. The same conditions were used as in Figure 2. The numbers above the peaks refer to rat GST subunit numbers.

Table 3 Separation of rat GST isoenzymes by novel sorbents

Sorbent	Isoenzyme subunit specificity	Protein recovery (%)
High binding, non-selective		
Hx-GSH	All	3.0
S-L-GSH	All	2.5
Selective		
γ E-C(Cu)-G	2 > 3 > 4	2.5
γ E-C(BuBz)-G	2 > 4 > 3 > 7	1.7
γ E-C(Bz)-A	4 > 3 $\gg$ 6 > 2	0.5
γ E-C(Bz)- β A	3 = 4 $\gg$ 2 > 6	1.0
γ E-C(Pr)-H	4 > 3 $\gg$ 2	1.0
γ E-C(Pr)-A	3 = 4 > 2 > 1 > 6 > 7	1.4
γ E-C(Hx)- ϕ G	7 $\gg$ 3 = 4	0.2
γ E-C(Bz)- ϕ G	7 $\gg$ 2 = 3 = 4	1.3
Weak binding		
γ E-C(Pr)-V	Little; 2 = 3 = 4	0.5
γ E-C(Hx)-V	Little; 2 = 3 = 4	0.4
γ E-C(Pr)-D	Little; 3 > 2 > 4	0.2
γ E-C(Hx)-D	None	0.1
γ E-C(Bz)-H	None	0.1

in the eluates from γ E-C(Bz)-A and γ E-C(Bz)- β A, but was not detected in eluates from the other sorbents. Interestingly, in our hands, HxGSH, previously shown to bind glyoxylase I (Hayes, 1988), did not show any detectable levels of this GSH-dependent enzyme. Glyoxylase I is known to be a metal-dependent enzyme, and the presence of EDTA in the buffers may have interfered with binding activity.

The high degree of purity of appropriately sized proteins seen by SDS/PAGE of the affinity-eluted proteins suggested that the observed bands are indeed GSTs. Independent confirmation of this conclusion, at higher resolution, was provided by RP-h.p.l.c. analyses of the GST isoenzymes in the eluates from different sorbents (Figure 2). Comparison with purified GST standards showed that the isoenzymes eluted from the novel sorbents correspond to known GST isoenzymes. These procedures showed that GST subunit 3 eluted at 14 min, subunit 4 at 17 min, subunit 7 at 18 min, subunit 2 at 23 min, subunit 6 at 25 min, subunits 1a and 1b at 37 and 40 min, and subunit 8 at 42 min. Subunits 3, 4 and 6 are Mu-class isoenzymes, subunits 1a, 1b, 2 and 8 are Alpha-class isoenzymes, and subunit 7 is the Pi-class isoenzyme. S-L-GSH and HxGSH sorbents, as expected, bound all except 5-5, the Theta-class isoenzyme. γ E-C(Cu)-G (Figure 2b) increased the proportion of isoenzyme 2, yet bound all the isoenzymes to some extent. In contrast, other sorbents were very

selective in their binding of the isoenzymes. For example, γ E-C(Bz)- β A (Figure 2c) selectively bound the Mu-class isoenzymes 3 and 4, confirming the SDS/PAGE results.

Use of the γ E-C(Hx)- ϕ G sorbent with liver cytosol suggested that this sorbent binds the Pi isoenzyme 7-7 with greater selectivity than does the HxGSH sorbent (Figures 3a and 3b). As noted above, we tested this sorbent with cytosol from kidney, lung and small intestine, tissues known to contain higher levels of isoenzyme 7-7. Figures 3(c) and 3(d) show the RP-h.p.l.c. analysis of the isoenzymes from kidney cytosol retained by HxGSH and γ E-C(Hx)- ϕ G respectively. From the wide variety of isoenzymes present in kidney cytosol, the new sorbent selectively bound only isoenzyme 7-7. These results were confirmed in both lung and small intestine (results not shown), indicating that γ E-C(Hx)- ϕ G is indeed highly selective for this isoenzyme.

The specificities of a variety of our novel sorbents for rat cytosolic GST isoenzymes are summarized in Table 3. Some structural alterations of the ligand produced sorbents that did not effectively bind any isoenzyme; several of these are listed for completeness.

DISCUSSION

The rat system has received extensive study with regard to GST biology and biochemistry. From these efforts, it is clear that resolution of isoenzymes is critical to a detailed understanding. For complete information, isoenzymes need to be isolated in active form, a goal not readily attainable by published methods. Previous work from this laboratory has shown that peptide-based ('paralogue') chromatography sorbents (Benedek et al., 1992) provide a rich new source of general purpose separation media. In this paper, the paralogue approach has been successfully extended to resolution of GST isoenzymes under non-denaturing conditions.

Adang et al. (1990) developed a general model for the GSH-binding site, or G-site, of the individual GST isoenzymes based on the ability of the isoenzymes to utilize GSH analogues as substrates. The γ -Glu moiety of GSH was found to be highly critical for activity (Adang et al., 1989). Modification of the L-Cys to D-Cys, L-homocysteine or L-penicillamine resulted in tripeptides that were poor substitutes for GSH, although the latter two inhibited CDNB-conjugating activity with GSH, indicating that they did bind to the G-site. Alterations of the Gly moiety resulted in substantial changes in specificity of the individual isoenzymes. Adang et al. (1990) also suggested that interactions occurred between the Cys and Gly substituents in the binding site and that modification of both moieties could lead to substrates with a higher CDNB-conjugating activity than that of GSH.

We used these results to begin our systematic modifications of GSH, concentrating on the Cys and Gly moieties. Substitution of phenylglycine for Gly, increasing the hydrophobicity and bulk of this site, resulted in high specificity for the Pi-class isoenzyme 7-7. Alteration of the S-linked moiety to a structure similar to that of cumene hydroperoxide, an Alpha-class substrate, made the sorbents preferential for isoenzyme 2-2 of the Alpha-class.

The use of Ala and β -Ala at the Gly site produced sorbents with high specificity for the Mu-class isoenzymes. The Ala sorbent was most specific for GST subunit 4. The shift from Ala to β -Ala, an alteration that increases the length of the backbone, resulted in increased binding of subunit 3, consistent with increased CDNB-conjugating activity with this analogue as substrate. The Gly substituents do not determine the total character of the sorbent, however. For example, the use of hexyl

at the Cys site, combined with Ala or β -Ala at the Gly moiety, resulted in no GST binding, as seen by h.p.l.c. analysis.

Systematic alterations of the Cys residue to probe the previously defined H-site (hydrophobic pocket) resulted in ligands capable of binding to the isoenzymes. Adang et al. (1990) did not examine any analogues with systematic modifications of groups attached to the Cys moiety. Alterations of this group had effects on the level of protein recovery and the specificity of isoenzyme binding in several cases. When Ala was used as the terminal amino acid, both propyl and benzyl moieties on the Cys resulted in sorbents specific for Mu-class isoenzymes (Table 3). The propyl sorbent retained more GST protein, in part because it picks up both isoenzymes 3 and 4, compared with the benzyl sorbent, which is highly specific for isoenzyme 4. When His was used as the terminal amino acid, the propyl moiety resulted in high Mu-class specificity, while the benzyl moiety resulted in no binding at all. In contrast to these sorbents, alterations of the Cys moiety of Val- and Asp-substituted sorbents had no effect on the low binding. Thus the Cys substituent can be important in modifying the overall specificity of the sorbent as determined by the terminal amino acid.

The ability of these novel sorbents to selectively purify active GST isoenzymes will have many applications in clinical and biochemical investigations. For example, P1-1, the human Pi-class isoenzyme, is increased in some cisplatin-, adriamycin- and chlorambucil-resistant human tumour cell lines and in cancer tissues (Batist et al., 1986; Lewis et al., 1988; Nakagawa et al., 1988), often to levels that can overwhelm the detection of other isoenzymes. This isoenzyme is also found in high levels in most growing tissues, such as the small intestine (Kelley and Bjeldanes, 1991) and placenta (Mannervik and Danielson, 1988). Thus the ability to specifically remove the Pi-class isoenzyme from cytosol with γ E-C(Hx)- ϕ G sorbent will result in an increased ability to examine other isoenzymes that may have a more critical impact on drug resistance, despite their low levels. For such purposes, the mild nature of the separation, which preserves activity, is particularly advantageous. The transfection of GST Pi genes into pT22-3 cells, for example, confers no significant increase in resistance to cisplatin, chlorambucil or melphalan (Tew et al., 1988), suggesting the importance of other isoenzymes. This hypothesis is supported by the work of Smith et al. (1989), who found decreased total activity against CDNB in a BCNU-resistant rat glioma cell line compared with a sensitive line, contrary to expectation; the discrepancy was resolved by isoenzyme characterization by laborious methods which showed a large decrease in levels of an abundant form, the Pi-class isoenzyme 7-7, and an increase in an isoenzyme capable of metabolizing BCNU, 4-4.

Hexyl-GSH sorbent has been used to separate glyoxylase I from the cytosol (Hayes, 1988). The new sorbents γ E-C(Bz)- β A and γ E-C(Bz)-A bind greater amounts of this enzyme, as shown by SDS/PAGE, than does the HxGSH sorbent, indicating that these novel sorbents may be useful for investigation of glyoxylase I as well as of other GSH-linked enzymes.

Changes in CDNB-conjugating activity and protein content in the eluates reflects the selection of specific isoenzymes by the different sorbents. The low level of protein and activity recovery from liver cytosol with the γ E-C(Hx)- ϕ G is due to its selection of the 7-7 isoenzyme, a minor component of liver, which furthermore has a lower specific activity towards CDNB than the other liver isoenzymes (Mannervik and Danielson, 1988). Conversely, γ E-C(Bz)-A retains 15% of the protein found with HxGSH, but because of the higher specific activity of isoenzyme 3, which is the major component of the γ E-C(Bz)-A eluate, the activity towards CDNB is reduced to only 33% of the level

found with HxGSH. The use of selective sorbents thus bypasses the inherent problems of relying upon enzyme activity assays for GST isoenzyme profile determination. The use of an inhibitory ligand as eluant leaves some residual uncertainty regarding isoenzyme activities, since not all isoenzymes are identically inhibited, but this factor can be largely compensated for, as described in the Materials and methods section, and is a smaller source of error than the differences in specificity for CDNB.

Meyer et al. (1991) have recently isolated isoenzymes of the Theta-class from rat and human tissues using Matrex Gel Orange A sorbent (Amicon, Beverley, MA, U.S.A.) followed by hydroxyapatite, anion-exchange and hydrophobic interaction chromatography. The rat 5-5 isoenzyme of the Theta-class is known to have high specific activity towards organic hydroperoxides and epoxyeicosatrienoic acids (Ketterer et al., 1988). Efforts to develop a GSH analogue sorbent that would selectively bind this class of isoenzymes have been initiated.

The use of flow-through microplates for affinity chromatography is a significant technological improvement over the use of standard mini-columns. This technique allows the rapid, simultaneous testing of multiple sorbents in replicates for more reliable quantification. Samples can easily be incubated in a standard refrigerator. Because the procedure can be accomplished with relatively small amounts of cytosol and sorbent, a single sample can be analysed by several sorbents with different specificities.

Chromatography has proven to be an efficient initial step in the characterization of the interactions of our novel ligands with the full variety of GST isoenzymes, only a few of which are readily available in purified form. Chromatography is also a versatile and sensitive technique for assessing molecular interactions (Fassina and Chaiken, 1987), particularly in the moderate binding affinity range expected for interactions of GSTs with analogues of glutathione, a cellular constituent normally present at or above 1 mM concentrations. The simplified selection of sorbents for more detailed study reported here may thus be expected to yield a wealth of data on the distinctive features of the binding sites of the various isoenzymes.

Given the immense variety of substrates and inhibitors known to bind to GST (Mannervik and Danielson, 1988) and the variety of possible N- and C-terminal alterations, the potential number of GSH-based ligands that could be created is in the thousands. Systematic sampling to achieve diversity in multiple characteristics (Kauvar, 1992) has proven to be an efficient short cut to identifying useful ligands for GSTs. Our initial work with systematic concurrent alterations of different moieties of GSH produced compounds selective for different GST isoenzymes in an affinity chromatography format. We are currently testing the applicability of these sorbents for the isolation and characterization of human GST isoenzymes from both normal and cancer tissues.

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