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Inhibition and recognition studies on the glutathione-binding site of equine liver glutathione *S*-transferase

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Equine liver glutathione *S*-transferase has been shown to consist of two identical subunits of apparent M_r 25500 and a pI of 8.9. Kinetic data at pH 6.5 with 1-chloro-2,4-dinitrobenzene as a substrate suggests a random rapid-equilibrium mechanism, which is supported by inhibition studies using glutathione analogues. *S*-(*p*-Bromobenzyl)glutathione and the corresponding N_α-, C_{Glu}- and C_{Gly}-substituted derivatives have been found, at pH 6.5, to be linear competitive inhibitors, with respect to GSH, of glutathione transferase. *N*-Acetylation of *S*-(*p*-bromobenzyl)glutathione decreases binding by 100-fold, whereas *N*-benzoylation and *N*-benzyloxycarbonylation abolish binding of the derivative to the enzyme. The latter effect has been attributed to a steric constraint in this region of the enzyme. Amidation of the glycine carboxy group of *S*-(*p*-bromobenzyl)glutathione decreases binding by 13-fold, whereas methylation decreases binding by 70-fold, indicating a steric constraint and a possible electrostatic interaction in this region of the enzyme. Amidation of both carboxy groups decreases binding significantly by 802-fold, which agrees with electrostatic interaction of the glutamic acid carboxy group with a group located on the enzyme.

INTRODUCTION

The glutathione *S*-transferase(s) (EC 2.5.1.18) are diverse proteins of cytosolic origin found in mammals and micro-organisms that are active in the binding of hydrophobic molecules, detoxification of xenobiotic compounds (Jakoby & Habig, 1980), including products of cytochrome *P*-450-dependent reactions, transport functions and possibly the isomerization of certain steroids (Benson *et al.*, 1977). They may also participate in the biosynthesis of leukotriene C_4 (Hammastrom *et al.*, 1979) and therefore play a role in the lipoxygenase pathway of arachidonic acid metabolism, afford micro-organisms resistance against antiprotozoal drugs (Penninckx & Jaspers, 1982) and act as a source of haem prosthetic groups for apo-cytochrome *P*-450 (Husby *et al.*, 1981).

These proteins catalyse conjugation reactions involving a single displacement mechanism (Mangold & Abdel-Monem, 1983) in which the activated thiol group of glutathione (GSH) undertakes a nucleophilic attack on the electrophilic centre of a compound (R-X) (Keen & Jakoby, 1978) to yield the corresponding water-soluble diastereomeric glutathione *S*-conjugate (Mangold & Abdel-Monem, 1983), which may be further metabolized to a mercapturic acid (i.e. *N*-acetylcysteine derivative).

These enzymes usually occur in multiple forms, and in mammalian tissues they have been tentatively grouped into three distinct classes named Alpha, Mu and Pi (basic, near-neutral and acidic respectively) on the basis of common substrate and inhibitor specificities, immunological properties, pI and amino acid sequence (Mannervik, 1985). Most transferases exist as dimers composed of identical (homodimers) or non-identical (heterodimer) subunits. Each subunit contains two substrate-binding sites: a non-specific site of hydrophobic nature (H-site; see Fig. 1) (Mannervik, 1985) utilized for the binding of a broad spectrum of electrophilic compounds (Habig *et al.*, 1974), and a second specific site for the binding of GSH (G-site) (Jakobson *et al.*, 1977), which may be replaced by homoglutathione (α-Glu-Cys-β-Ala) (Habig *et al.*, 1974), but not 2-propylthiouracil (Habig *et al.*, 1984).

Attempts to characterize both these sites have been hindered by weak binding constants for electrophilic substrates and GSH.

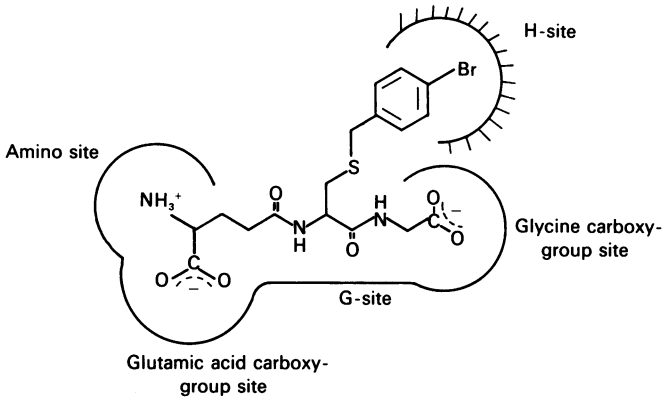


Fig. 1. Nomenclature of binding subsites for glutathione derivatives

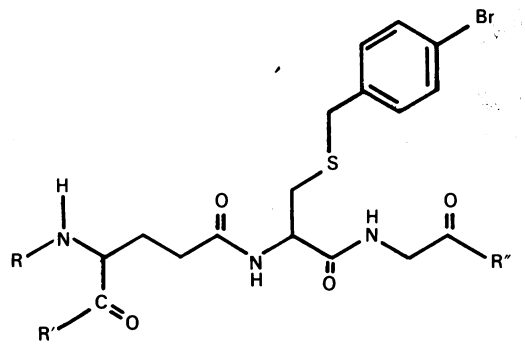
To overcome these limitations and obtain information on the GSH-binding site, especially in view of its importance to catalysis, we have exploited the high affinity of *S*-blocked glutathiones, which are product analogues. Derivatives modified alternatively at the amino and carboxy functional groups were prepared, and detailed inhibition studies were undertaken, aimed at probing the G-site of equine liver glutathione *S*-transferase.

MATERIALS AND METHODS

GSH and equine liver glutathione transferase purified according to the method of Simons & Vander Jagt (1977) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. This enzyme preparation had an activity of 83 units/mg of protein using 1-chloro-2,4-dinitrobenzene (CDNB), 1.9 units/mg of protein using 4-nitrobenzyl chloride and 0.11 unit/mg of protein using 1,2-epoxy-3-(4-nitrophenoxy)propane as substrates. All other chemicals were standard commercial products. GSH derivatives 1–3 (see Table 1) were prepared as described in D'Silva *et al.* (1982) and **4–8** as described in D'Silva [1986, 1990 (the following paper)].

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; 4-Abu, 4-aminobutyric acid.

Table 1. Inhibition of equine glutathione transferase by amino and carboxylic-substituted *S*-blocked glutathione derivatives in phosphate buffer, pH 6.5, at 25 °C



Compound	R	R′	R″	K_i (μM)*	Ratio†
1	–H	–OH	–OH	0.8±0.11	1
2	$-COCH_3$	–OH	–OH	80±20‡	100
3	$-COC_6H_5$	–OH	–OH	n.d.§	
4	$-CO_2CH_2C_6H_5$	–OH	–OH	n.d.§	
5	–H	–OH	$-OCH_3$	55.7±7.7	70
6	–H	–OH	$-NH_2$	10.3±1.1	13
7	–H	$-OCH_3$	$-OCH_3$	1035±16	1294
8	–H	$-NH_2$	$-NH_2$	642±30‖	802

* Prepared in dimethyl sulphoxide [concn. < 1% (v/v)].
† The ratio is the K_i value of derivatives **2**–**8** over the K_i value of derivative **1**.
‡ Prepared in dimethyl sulphoxide [concn. 3.33% (v/v)].
§ n.d., no detectable binding.
‖ Prepared in water.

The apparent M_r of equine liver transferase was determined by gel filtration on a calibrated Pharmacia Superose 12 column eluted with 0.05 M-potassium phosphate buffer, pH 7.5, and the subunit M_r was estimated by SDS/PAGE (Laemmli, 1970). Protein concentrations were determined by the method of Lowry *et al.* (1951), with BSA as standard. Analytical isoelectric focusing was carried out over the pH range 3.5–9.5 on LKB Ampholine PAG gels. Separations were run at 350 V for 18 h, then at 800 V for 3 h, and stained with Coomassie Brilliant Blue R-250.

Kinetic methods

Glutathione transferase activity was measured at 25 °C in 0.1 M-potassium phosphate buffer (pH 6.5)/1 mM-EDTA/1.66% (v/v) ethanol/GSH (0.03–5.0 mM)/CDNB (0.01–0.5 mM) in a final volume of 3.0 ml. After pre-incubation at 25 °C, the non-enzymic rate was measured and the reaction initiated by addition of 20–30 μl of equine glutathione transferase to give a final concentration of 0.55–0.1 μg/ml. The catalytic reaction was monitored at 340 nm, and the activity in units was calculated after correction for the non-enzymic rate using ϵ_{340} 9.6 $mM^{-1} \cdot cm^{-1}$ (Habig *et al.*, 1974).

Inhibition studies

Inhibition by GSH derivatives (**1**–**5**; see Table 1) of the transferase-catalysed conjugation of CDNB with GSH was determined at 25 °C and at pH 6.5 by monitoring the initial rate of product formation at 340 nm of a fixed concentration of CDNB (1 mM) and various levels of GSH at at least three inhibitor concentrations. The procedure was then repeated using a fixed concentration of GSH (1 mM) and various levels of CDNB at several fixed concentrations of inhibitor. Inhibition was analysed by Lineweaver–Burk $1/v_0$ versus $1/s_0$ plots and the corresponding slope and intercept replots. Computations were performed on a Vig II/AT microcomputer using least-squares linear-regression analysis programs.

RESULTS

Molecular and kinetic properties

Equine liver glutathione transferase prepared according the method of Simons & Vander Jagt (1977) exhibits a comparably high specific activity with CDNB, 4-nitrobenzyl chloride and 1,2-epoxy-3-(4-nitrophenoxy)propane compatible with specific activities and substrate specificities reported for rat liver glutathione transferase A (Habig *et al.*, 1974) and human liver transferase μ (near-neutral) and α–ε (basic) (Kamisaka *et al.*, 1975; Warholm *et al.*, 1983). The apparent M_r determined by gel filtration on a Pharmacia Superose 12 column for this enzyme was 52000, the basis of interpolation of linear plots of log M_r versus R_F, using cytochrome *c* (M_r 13000), chymotrypsin (25000), BSA (M_r 65000) and γ-globulin (M_r 150000) as standards. SDS/PAGE gave a single band with a subunit M_r of 25500 using lysozyme (M_r 14300), soya-bean trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r 43000), BSA (M_r 66000), and phosphorylase *b* (M_r 94000) as standards. This result is consistent with the enzyme being a homodimer composed of two identical subunits. The pI determined for this enzyme preparation by isoelectric focusing in a pH gradient of 3.5–9.5 was centred at 8.9. The pH-dependence for the conjugation reaction between GSH and CDNB showed a bell-shaped profile with the optimum centred at approx. pH 7.4 (Fig. 2).

The steady-state kinetics with varied GSH or CDNB, studied at pH 6.5 with a fixed concentration of the other, gave linear double-reciprocal plots with a common intersection point on the abscissa in both cases (see Figs. 3*a* and 3*b*). Intercept replots of the data were linear in both cases and did not show the deviation from Michaelis–Menten kinetics that was observed with rat liver transferase A (3-3; Yb_1Yb_1) when [GSH] is varied (Pabst *et al.*, 1974; Askelof *et al.*, 1975; Jakobson *et al.*, 1977) or with a non-homogeneous preparation of enzyme. Analysis of the experimental data in terms of the initial-rate equations for a two-

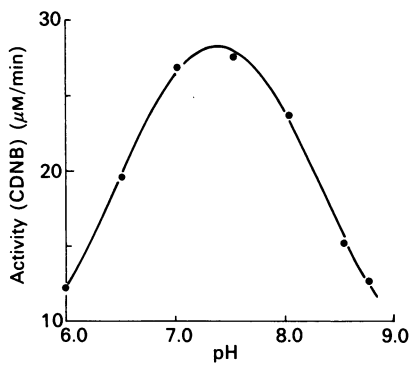


Fig. 2. pH-dependence of the initial velocities obtained with CDNB (1 mM) and GSH (1 mM) in potassium phosphate/pyrophosphate buffer (0.1 M) containing EDTA (1 mM), at 25 °C

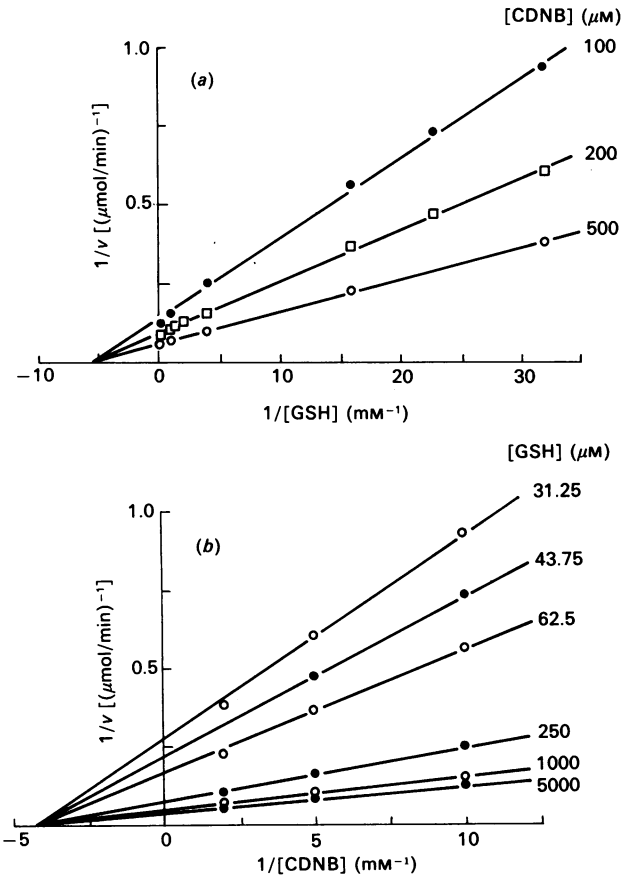


Fig. 3. Substrate kinetics for the reaction between CDNB and glutathione catalysed by equine glutathione transferase

Double-reciprocal plots of $1/v$ versus 1/[GSH] or 1/[CDNB] are presented in (*a*) and (*b*) respectively at various fixed concentrations of CDNB or GSH (pH 6.5, 25 °C).

substrate (A, B) sequential reaction (eqn. 1) gave Michaelis constants (K_a and K_b) of 192.6±30 μM and 288.4±27 μM for GSH and CDNB respectively:

$$v = \frac{V_{max.}[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

The apparent kinetic dissociation constants (K_{ia} and K_{ib}) were similar, with mean values of 182±5.5 μM and 241±41 μM for GSH and CDNB respectively. The maximum rate of catalysis was determined as 2976±357 min^{-1}.

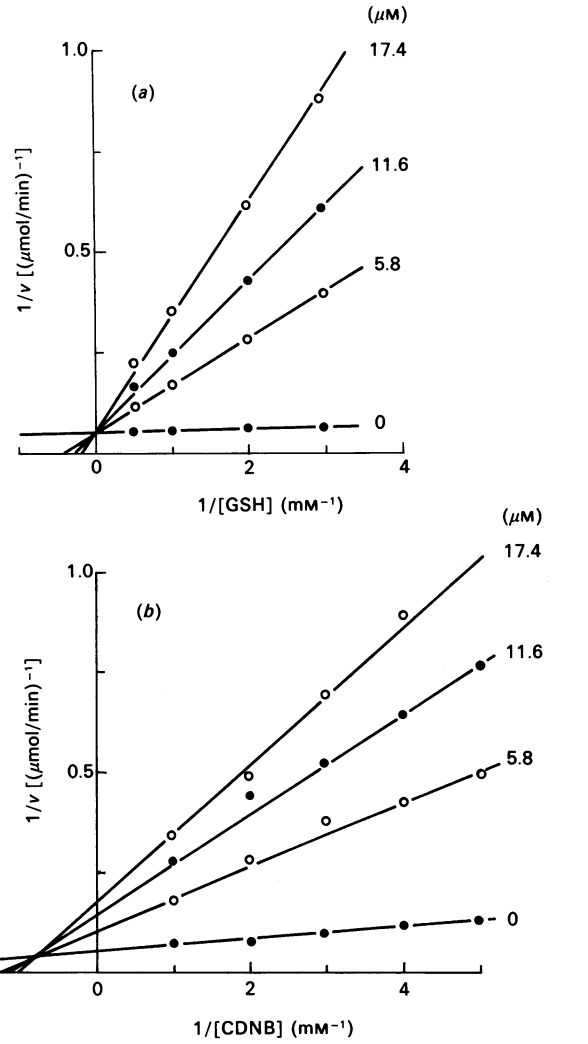


Fig. 4. Inhibition of glutathione transferase by S-(p-bromobenzyl)-glutathione with (*a*) GSH and (*b*) CDNB as the variable substrate

Double-reciprocal plots of $1/v$ versus 1/[GSH] or 1/[CDNB] are presented in (*a*) and (*b*) respectively at various fixed concentrations of inhibitor used. The fixed substrate was maintained at 1 mM in both experiments.

Inhibition of glutathione S-transferase by S-substituted GSH derivatives

Inhibition studies using the S-blocked glutathione derivative S-bromobenzylglutathione (**1**) gave linear-competitive kinetics with respect to glutathione ($K_i = 0.8$ μM) and the slope and intercept indicated linear/mixed-type non-competitive inhibition with respect to CDNB ($K_{is} = 1.7$ μM; $K_{ii} = 15.8$ μM) at pH 6.5 and 25 °C (see Fig. 4).

The *N*-acyl derivatives **2**, **3** and **4**, amide derivatives **6** and **8** and ester derivatives **5** and **7** were weaker inhibitors of glutathione transferase than was derivative **1** (see Table 1), but all were found to be linear competitive inhibitors with respect to glutathione.

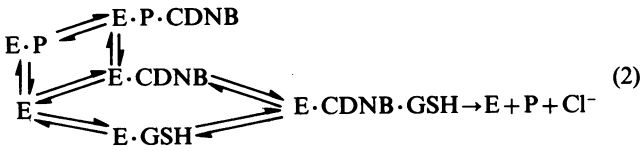
DISCUSSION

Equine liver glutathione transferase was found to be a homogeneous enzyme preparation by the criterion of PAGE, isoelectric focusing and the linearity of primary plots used to determine the kinetic constants K_a, K_b and K_{ia}. This enzyme is a homodimer composed of two identical subunits of apparent M_r 25500, each of which contains two binding sites: one subsite for GSH (G-site) and its derivatives, and one for the electrophilic substrate (H-

site) (Mannervik, 1985). The transferase studied here is much larger than the isoenzyme isolated from equine erythrocytes by Ricci *et al.* (1989) using the purification procedure of Simons & Vander Jagt (1977). This enzyme was a non-symmetrical homodimer immunologically related to the Pi class of transferase, with an M_r 48000 and a pI of 5.9. The enzyme studied here shares similarities in substrate specificity and pI to the basic transferases α–ϵ (Kamisaka *et al.*, 1975; Warholm *et al.*, 1983), which occur in separable, but functionally similar, forms.

Kinetic studies on glutathione transferase give a kinetic pattern consistent with eqn. (1) above. The common intersection point on the abscissa of the double-reciprocal plots in Figs. 3(*a*) and 3(*b*) and the similarity in magnitude of the Michaelis constants (K_a and K_b) and the kinetic dissociation constants (K_{ia} and K_{ib}) collectively indicate that the enzyme subunits are catalytically equivalent and that the binding of GSH has no effect on the binding of CDNB and vice versa. The apparent Michaelis constants (K_a or K_b) for the first substrate are equal to the true dissociation constant only if the kinetic mechanism is a random rapid-equilibrium one.

An ordered mechanism can be distinguished from a random system by product-inhibition studies (Segal, 1975), assuming the Cl^- ion is released before the product. The product, *S*-(2,4-dinitrophenyl)glutathione, and the product analogue, *S*-(*p*-bromobenzyl)glutathione (see Fig. 4), were prepared and both shown to be competitive inhibitors with respect to GSH and non-competitive inhibitors with respect to CDNB. For the random rapid-equilibrium mechanism the product is expected to have the greatest affinity for the free enzyme and therefore be competitive with respect to both substrates. The observed non-competitive inhibition with respect to CDNB can be accommodated in the random rapid-equilibrium mechanism if the product (P) binds to the E·CDNB form of the enzyme (eqn. 2) (Segal, 1975):



(2)

This is not unlikely, as CDNB is bound in a non-specific hydrophobic binding site (H-site) located on the enzyme. The pattern of inhibition observed here was also reported for the GSH analogues γ-glutamylalanylglycine and γ-glutamylseryl-glycine (Chen *et al.*, 1985), which may be expected to bind to both the free enzyme and E·CDNB complex. In summary, the observed kinetic and inhibition pattern is consistent with an enzyme having two catalytically equivalent non-co-operative subunits in which the binding of one substrate does not affect the binding constant of the second and functioning by a random rapid-equilibrium mechanism (eqn. 2), as this is the simplest one consistent with the experimental results. Previous studies with glutathione transferase gave results consistent with an ordered mechanism with GSH adding first (Pabst *et al.*, 1974; Gillham, 1973) and for a Ping Pong mechanism at low GSH concentration (Pabst *et al.*, 1974). The results reported here are more in agreement with the random order of substrate addition proposed for glutathione transferase A (Mannervik & Askelof, 1975), μ (Warholm *et al.*, 1983), the Ya homodimer (Schramm *et al.*, 1984) and the enzyme from bovine brain (Young & Briedis, 1989). The ratio $k_{cat.}/K_m$ for CDNB is 1×10^7 $min^{-1} \cdot M^{-1}$, which is similar to the value of 1.8×10^7 $min^{-1} \cdot M^{-1}$ reported for glutathione transferase μ (Warholm *et al.*, 1983).

The product analogue *S*-(*p*-bromobenzyl)glutathione (**1**) was found to be a potent inhibitor of this enzyme, with $K_i \sim 1$ μM. The patterns of inhibition obtained with this derivative and *S*-(2,4-dinitrophenyl)glutathione are consistent with the random rapid-equilibrium mechanism proposed in which the inhibitor competes competitively against GSH but allows CDNB to bind. The simple kinetic mechanism and the competitive inhibition obtained with respect to GSH using derivative **1** permitted the studies proposed at probing the GSH-binding site of this enzyme.

Modification of the amino group of the *S*-blocked GSH derivative **1** by *N*-acetylation (**2**) significantly reduces binding, but does not totally abolish binding to the enzyme. The 100-fold increase in K_i in going from a free $-NH_3^+$ group (**1**) to the *N*-acetyl group (**2**) clearly indicates a significant contribution of the *N*-site to the overall binding of *S*-blocked glutathione inhibitors with equine liver glutathione transferase. The loss of binding on benzoylation or benzyloxycarbonylation (derivatives **3** and **4**) indicates the presence of a large steric effect at the *N*-site. This effect may account for the increased K_i observed on *N*-acetylation (compare **1** with **2**) and is indicative of the *N*-site being located in a pocket within the enzyme instead of being on the surface. The steric component, however significant, does not prevent binding of the *N*-acetyl derivative **2** and so accounts for only part of the binding energy lost. The remainder therefore must reflect the loss of interaction between the NH_3^+ form of the inhibitor and the *N*-site, assuming the pK_a of the amino group [9.34 for *S*-methylglutathione (Ball & Vander Jagt, 1981)] is not altered on binding. The difference in free energies of *N*-acetylated (compound **2**) and the NH_3^+-free (compound **1**) inhibitor is estimated as 11.4 $kJ \cdot mol^{-1}$ (2.7 $kcal \cdot mol^{-1}$) at 25 °C, using $\Delta\Delta G = \boldsymbol{R}T \cdot \ln \Delta pK_i$ [ΔpK_i is the ratio (100) of K_i values (see Table 1); G, $\boldsymbol{R}$ and T have their usual meanings]. This agrees with electrostatic binding at the *N*-site, presumably due to the formation of a salt linkage with an enzyme carboxylate ion in a region of low dielectric constant, consistent with a site located in a pocket within the enzyme. In agreement with these studies the free thiol derivative of **4** is not a substrate of equine glutathione transferase, probably owing to poor binding. Studies by Adang *et al.* (1988, 1989) on the substrate specificity of the isoenzymes of rat liver glutathione transferase have shown the deamino form of GSH to be a weak substrate, exhibiting catalytic efficiencies ($k_{cat.}/K_m$) less than 6.5% that of GSH. The low activity observed for the deamino form of GSH may reflect the loss of an electrostatic interaction which may be common to both the equine and rat liver enzymes. However, the presence of a steric constraint in this region of the enzyme may not be so general, as the purification of the isoenzymes of rat lung glutathione *S*-transferase, of which three isoenzymic forms are immunologically related to the rat liver isoenzymes, have been effected by affinity chromatography on a column prepared by coupling the α-amino group of *S*-hexylglutathione to epoxy-activated agarose (Guthenberg & Mannervik, 1979).

In contrast with equine liver glutathione transferase, the *N*-sites of yeast and erythrocyte glyoxalase I (Douglas *et al.*, 1982; Al-Timari & Douglas, 1986*a*) and bovine liver glyoxalase II (Al-Timari & Douglas, 1986*b*) are located on the surface of the enzyme, as minimal steric effects are observed with increasing size of *N*-substituents.

Amidation of the glycine carboxy group of the *S*-blocked glutathione derivative (**1**) does not abolish binding to the enzyme. The 13-fold decrease in K_i in going from the free glycine carboxy group (**1**) to the $CONH_2$ group (**6**) clearly indicates that this group has some contribution to binding. Both groups are similar in size and capable of H-bonding with groups on the enzyme. The decrease in binding must therefore reflect the loss of an electrostatic interaction between the carboxy group and a group located on the enzyme. The estimated binding energy for this electrostatic interaction is 6.3 $kJ \cdot mol^{-1}$ (1.5 $kcal \cdot mol^{-1}$) at 25 °C, on the basis of the difference in K_i of 13 between **1** and **5** (see Table 1). For an electron and proton in water separated by

0.33 nm (3.3 Å), the calculated interaction energy is 5.4 kJ·mol^{-1} (~ 1.3 kcal·mol^{-1}) (Fersht, 1977), which increases as the local dielectric constant (D) increases. This agrees with electrostatic binding of the glycine carboxy to a group located near or at the surface of the enzyme. The 5-fold decrease in K_i in going from the glycine $CONH_2$ group (**6**) to the CO_2CH_3 group (**5**) clearly reflects the loss of interaction due to steric effects caused by the larger size of the CO_2CH_3 group. However, this loss in electrostatic interaction does not prevent the free SH derivative of **5** being a weak substrate for equine and rate liver glutathione transferases (Inoue *et al.*, 1981).

Adang *et al.* (1989), using glycine-modified glutathione analogues to probe the GSH site of rat liver glutathione *S*-transferase, showed that the glycine group could be replaced by a variety of groups, such as β-alanine, 4-aminobutyric acid (4-Abu), L-alanine, L-phenylglycine, L-aspartic acid and $-NHC_2H_5$, and that these compounds were weak substrates for the 7-7 isoenzyme, exhibiting specific activities between 5 and 63% of that of GSH, whereas the same compounds were predominantly inhibitors of the 8-8 isoenzyme. The significant accommodation observed for a wide variety of groups at the glycine-carboxy-group site of rat liver glutathione transferase is consistent with this site being located on the surface of the enzyme, as ascertained from studies on equine glutathione *S*-transferase.

The explanation proposed is consistent with studies undertaken on yeast glyoxalase I (D'Silva, 1986), where an H-bond was proposed to exist between the glycine carboxy group and a group on the enzyme, on the basis of the absence of a difference in K_i between derivatives **1** and **6**.

Amidation of both the glutamic acid and glycine carboxy groups of the *S*-blocked glutathione derivative (**1**) significantly decreases binding to the enzyme. The 802-fold decrease in K_i (compare **1** with **8**) represents an electrostatic interaction energy of 16.5 kJ·mol^{-1} (~ 3.9 kcal·mol^{-1}) at 25 °C, on the basis of a difference in K_i of 802-fold between **1** and **8** (see Table 1). Substituting into eqn. (3) we can calculate an interaction energy for $\Delta\Delta G_{Glu}$ of 10.2 kJ·mol^{-1} (~ 2.4 kcal·mol^{-1}) at 25 °C. This result agrees with electrostatic interaction of the glutamic acid carboxy group with a cationic group located within the enzyme in a region of low dielectric constant (Fersht, 1977):

$$\Delta\Delta G_{Glu+Gly} = \Delta\Delta G_{Glu} + \Delta\Delta G_{Gly} \quad (3)$$

The interaction energy calculated for the glutamic acid carboxy-group site is similar in magnitude to that observed for the same site on yeast glyoxalase I (D'Silva, 1986) and clearly indicates the importance of this group to the binding of GSH derivatives in both enzymes. Consistent with these studies the decarboxy derivative of GSH (4-Abu-L-Cys-Gly) shows no activity as a substrate with rat liver glutathione transferase (Adang *et al.*, 1989).

In summary, N_α-, C_{Glu} and C_{Gly} *S*-blocked GSHs are useful derivatives in the study of the GSH-binding site of glutathione transferase. We infer from these studies that the amino and glutamic acid and glycine carboxy-group sites located on the enzyme are involved in the binding of GSH derivatives via electrostatic linkages and that the latter two sites are not equivalent, contributing unequally to the binding of *S*-blocked GSH derivatives. We also infer that the amino and glutamic acid carboxy-group site are located in a pocket within the enzyme, whereas the glycine carboxy-group site is at, or near, the surface of the enzyme. A possible candidate for electrostatic interaction with both the glutamic acid and glycine carboxy groups is arginine residues located on the enzyme (Schasteen *et al.*, 1983).

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